

*Salmonella* Suppress Innate Immunity by Targeting Mast Cells

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy in the  
Department of Pathology in the Graduate School  
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ABSTRACT

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## Abstract

Mast cells (MCs) are increasingly recognized as powerful sentinel cells responsible for modulating the early immune responses to a wide range of infectious agents. This protective role is attributable in part to their preponderance at the host-environment interface and their innate capacity to rapidly release modulators of immune cell trafficking, which promote the early recruitment of pathogen-clearing immune cells from the blood. However, host-adapted pathogens have been a critical threat to humans for a long time because they have evolved mechanisms directed at overcoming protective immunity.

In this work, we outline how *Salmonella enterica* serovar Typhimurium has evolved a novel mechanism to inactivate peripheral MCs, resulting in limited neutrophil responses at infection sites during early stages of infection. Due to the delay in bacterial clearance at the point of entry, *Salmonella* are able to multiply and rapidly disseminate to distal sites. Suppression of local MC degranulation restricts the outflow of vascular contents into sites of infection, thus facilitating bacterial spread.

We discovered that MC suppression is mediated by the *Salmonella* Protein Tyrosine Phosphatase (SptP), which shares structural homology with YopH, an effector protein expressed by plague-causing *Yersinia pestis*. Interestingly, SptP also shares homology with phosphatases found in MCs, and these phosphatases are also homologous to YopH. We show that YopH possesses the ability to suppress MCs like SptP, suggesting that this activity is common among some of the more virulent bacterial pathogens. The functionally relevant domain in SptP is its enzymatic site, which works by dephosphorylating the vesicle fusion protein N-ethylmaleimide-sensitive factor (NSF) and by blocking phosphorylation of Syk, which are located downstream and upstream of tyrosine phosphorylation signaling pathway in MCs, respectively.

Without SptP, oral challenge with *S. Typhimurium* fails to suppress MC degranulation and exhibits limited colonization of the mesenteric lymph nodes. Administration of SptP to sites of *Escherichia coli* infection markedly enhances its virulence. Thus, SptP-mediated inactivation of local MCs is a powerful mechanism utilized by *S. Typhimurium* to impede early innate immunity. This finding provides a logical explanation for why previous attempts by others to demonstrate a protective role for MCs against *Salmonella* infection have given equivocal results.

Taken together, this work highlights an overlooked virulence mechanism possessed by certain host-adapted pathogens to avoid the host innate immune system. Additionally, this innate immune-quelling property of SptP may hold future promise for tempering harmful inflammatory disorders in immune competent hosts.

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## List of Abbreviations

Abbreviations	
<b>ATP</b>	Adenosine Triphosphate
<b>BMMC</b>	Bone Marrow derived Mast Cell
<b>C48/80</b>	Compound 48/80
<b>CFU</b>	colony forming unit
<b>DAG</b>	Diacylglycerol
<b>Da</b>	Dalton
<b>dsRNA</b>	double-stranded ribonucleic acid
<b><i>E. coli</i></b>	<i>Escherichia coli</i>
<b>ER</b>	endoplasmic reticulum
<b>GFP</b>	Green Fluorescent Protein
<b>GTP</b>	Guanosine-5'-triphosphate
<b>GAP</b>	GTPase activating protein
<b>HIV</b>	Human Immunodeficiency Virus
<b>iNOS</b>	intrinsic oxidase synthase
<b>IL</b>	InterLeukin
<b>IP3</b>	inositol trisphosphate
<b>i.p.</b>	intraperitoneal
<b>ITAM</b>	Immunoreceptor Tyrosine-based Activation Motifs
<b>JNK</b>	c-Jun N-terminal kinase
<b>LAT</b>	Linker for activation of T cells
<b>LPS</b>	Lipopolysaccharides
<b>ManLAM</b>	Mannose-containing glycoprotein
<b>MAPKK</b>	Mitogen-activated protein kinase kinase
<b>MOI</b>	Multiplicity of Infection
<b>Mφ</b>	Macrophage
<b>MPO</b>	Myeloperoxidase
<b>ND</b>	Not determined
<b>NK</b>	Natural Killer
<b>NSF</b>	N-ethylmaleimide-Sensitive Factor
<b>PBS</b>	Phosphate buffered saline
<b>PLCγ</b>	Phospholipase γ
<b>PI3P</b>	Phosphatidylinositol 3-phosphate
<b>PKC</b>	Protein Kinase C
<b>PRR</b>	Pattern recognition receptor
<b>PTP</b>	Protein Tyrosine Phosphatase
<b>PTP-MEG2</b>	Protein Tyrosine Phosphatase Megakaryocyte 2

<b>Abbreviations</b>	
<b>SCV</b>	<i>Salmonella</i> Containing Vesicle
<b>SNARE</b>	Soluble NSF (N-Ethylmaleimide-Sensitive Factor) Attachment Protein Receptor
<b>SPI</b>	<i>Salmonella</i> pathogenicity islands
<b>SptP</b>	<i>Salmonella</i> Protein Tyrosine Phosphatase
<b>SipB</b>	<i>Salmonella</i> Invasion Protein B
<b>STm</b>	<i>Salmonella</i> Typhimurium
<b>TAT</b>	Transactivator of Transcription
<b>TLR</b>	Toll-like receptor
<b>TNF</b>	Tumor necrosis factor
<b>T3SS</b>	Type III secretion system
<b>WT</b>	Wild-type

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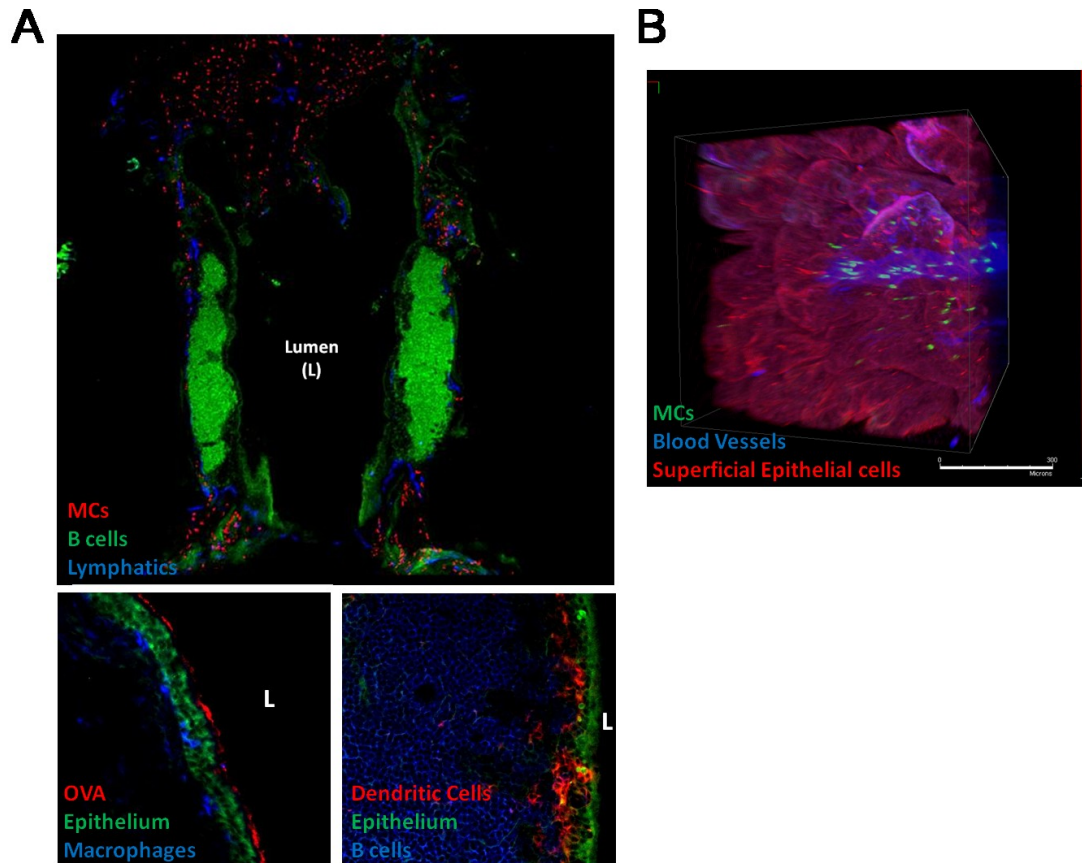
# **1. Introduction to Mast Cells and *Salmonella***

## **1.1 Mast Cell Regulation of Innate Immunity.**

### ***1.1.1 Mast cells as Pivotal Immune Surveillance Cells***

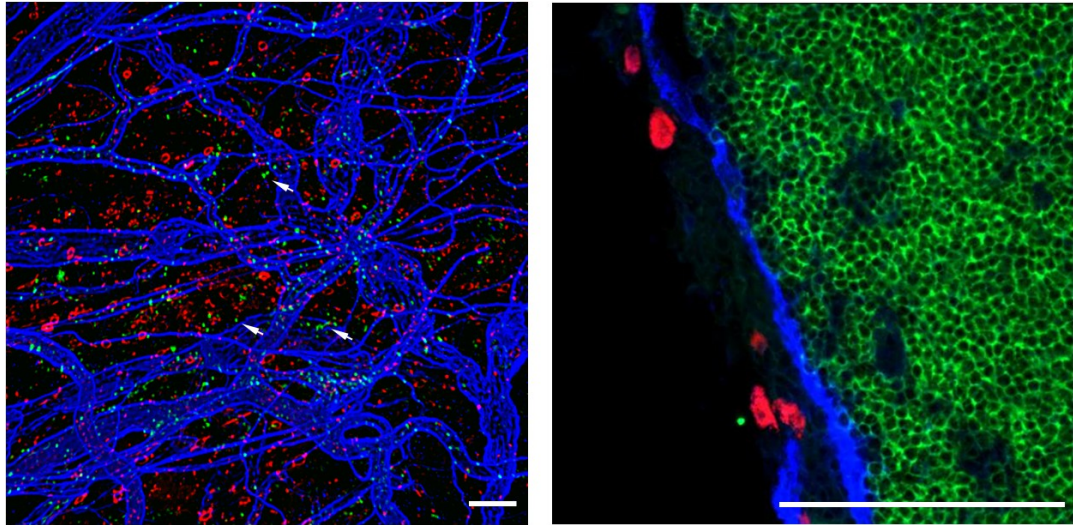
After heart diseases, microbial infections are the second most common cause of human death in the world [2]. MCs have several innate properties that make them uniquely able to contribute to immune surveillance against infectious agents. These properties include their selective location in relatively large numbers immediately underneath the epithelial surface of the skin and the mucosa of the genitourinary tract and respiratory tract (Figure 1). Because these sites are typically where infections begin, MCs appear strategically located to be among the first immune cells that pathogens encounter after they have breached the epithelial barrier. MCs are also found in close proximity around peripheral blood and lymphatic vessels (Figure 2), and thus they are also ideally positioned to recruit immune cells from blood vessels into infected tissue or coordinate the movement of immune cells from infected sites into lymphatic vessels leading to the draining lymph nodes [3,4]. Recently, it was reported that MCs proximal to blood vessels constantly sample blood contents by employing cytoplasmic protrusions [5] suggesting that these cells are also capable of detecting and interacting with blood borne pathogens.





**Figure 1. Strategic location of mast cells at the host-environment Interface**

(A) Nasal passage from mouse was dissected and cryosectioned longitudinally. (Upper panel) MCs, B cells, and lymphatics were stained with avidin, anti-B220, and anti-LYVE-1 antibodies, respectively. (Bottom left panel) Macrophages were stained with anti-F4/80 antibody. Respiratory epithelial cells were visualized with autofluorescence and covered with fluorescent ovalbumin by nasal challenge. (Bottom right panel) DCs and B cells were stained with anti-CD11c and anti-B220 antibodies. Respiratory epithelial cells were also visualized with autofluorescence. L indicates airway lumen. (B) Bladder tissue was dissected and whole-mount-stained with wheat germ agglutinin, avidin, and anti-CD31 antibody for superficial epithelial cells, MCs, and blood vessels, respectively. The image is a 3D reconstruction view from Z-stacked images taken by confocal microscope. Scale bar: 300  $\mu$ m.



**Figure 2. MC proximity to blood and lymphatic vessels.**

Abundant MCs are found near blood vessels in the mouse ear (left). Red fluorescence identified cells are MCs stained with avidin. Blue represents blood vessels stained with anti-CD31 antibody. Green depicts fluorescent dextran (150 kDa). When these MCs undergo degranulation, they induce blood vessel leakage that can be detected by the exudation of fluorescent dextran from blood vessels, indicated by green particles located in interstitial spaces, arrow. MCs are also typically found near lymphatics (right). Blue depicts lymphatic vessels stained for LYVE-1. Green depicts B cells stained with anti-B220 antibody. Red depicts MCs stained with avidin. Scale bar: 10  $\mu\text{m}$

### ***1.1.2 Mast Cells Respond Rapidly to Invading Pathogens Through Degranulation.***

MCs are highly specialized for the synthesis and secretion of a myriad of pharmacologically-active products. MC mediators have traditionally been divided into two major groups: those that are preformed, which include histamine, heparin, serine proteases, some select cytokines and chemokines such as tumor necrosis factors (TNFs) [6] and CXCL1/CXCL2 [7], and those that are synthesized *de novo* when the cells are activated, which include a wide range of cytokines and eicosanoids such as leukotrienes, prostaglandins and the thromboxanes [8]. The release of pre-packaged mediators into the surrounding tissue typically occurs beginning within seconds to minutes following stimulation, a strategy that gives MC-derived products a temporal advantage over those produced by other immune surveillance cells, which take markedly longer. The prepackaged MC products can be released in an explosive fashion or piecemeal.

Piecemeal degranulation consists of a slow emptying of granule chambers in the absence of large scale intergranular fusion. Because of their longevity, MCs at sites of inflammation can undergo multiple cycles of degranulation followed by re-granulation; in this way MCs are able to sustain inflammatory responses to pathogens. Unlike the degranulation response, which is initiated within seconds of MC activation, *de novo* synthesis of eicosinoids, cytokines and chemokines occurs significantly later, with a time frame that is comparable to when other immune cells secrete their mediators following activation. Many studies have shown that *de novo* production of these mediators by MCs in response to pathogens can vary greatly depending on the stimulus and the experimental conditions.

### ***1.1.3 Mast Cells Express a Wide Array of Receptors that Recognize a Broad Range of Pathogens.***

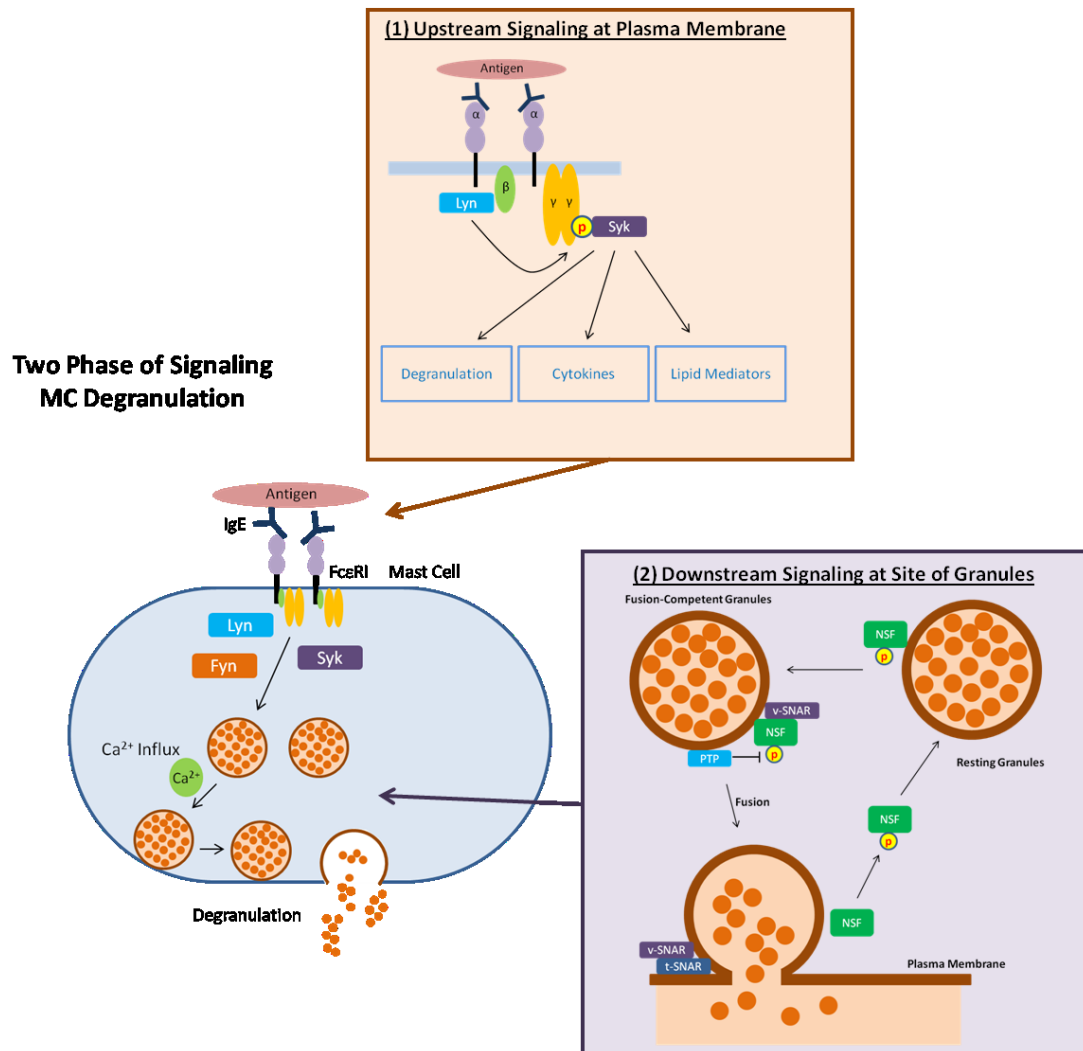
Like other immune cells involved in immune surveillance, MCs express a wide range of cell surface receptors that can directly bind pathogens, secreted toxins, or other microbial products. These receptors include most pattern recognition receptors (PRRs) as well as other receptors that specifically recognize microbial cell surface components. One of the best studied bacterial receptors on MCs is CD48, a GPI-anchored protein that binds type I fimbriae on various enterobacteria and a cell surface component on *Mycobacterium tuberculosis* [9,10]. Cross-linking of CD48 not only causes MC degranulation but also uptake of adherent bacteria. Additionally, MCs also express receptors on their cell surface for host proteins that coat pathogens, such as complement fragments and antibodies [11]. Depending on the receptor that is engaged, their cross-linking can trigger extensive or piece-meal MC degranulation. For example, cross-linking of CD48 by type I fimbriated *E. coli* can result in extensive MC degranulation and release of a wide range of mediators, whereas activation of Toll-like receptor (TLR) 4 by lipopolysaccharide (LPS) results in the significant secretion of soluble mediators but little or no MC degranulation. Similarly, binding of cholera toxin to the ganglioside GM1 results in the significant secretion of interleukin-6 (IL-6) but no accompanying degranulation of MCs [12].

MC activation during infection is not always the result of contact with an infectious agent, its products or with opsonized microbes; many infected or otherwise stressed cells release danger signals or alarmins that serve as potent activators of MCs [13,14]. Therefore, MCs do not have to be in immediate proximity to a site of infection to contribute to the immune response. The signaling agents released by stressed host cells range from antimicrobial peptides and adenosine triphosphate (ATP) to IL-33, which is typically released when cells degrade [15]. Binding of any of these cellular products to MCs can trigger extensive MC degranulation.

#### ***1.1.4 Signaling Events in Mast Cells that Regulate Mast Cell Exocytosis.***

Despite the large body of information regarding immune recognition receptors for pathogens or their products found on MCs, the question of how MCs signal intracellularly to induce a secretory response remains largely unknown. However, several key signaling events are likely shared with the well-characterized IgE signaling pathway, which is triggered when allergens bind and crosslink IgE bound to the high-affinity IgE receptor (FcεRI) on MCs [16]. As Figure 3 demonstrates, when FcεRI receptors aggregate, they initiate a cascade of tyrosine phosphorylation events via multiple signaling molecules in the MC cytoplasm. Early on, activated Lyn kinase phosphorylates tyrosine residues at immunoreceptor tyrosine-based activation motifs (ITAMs) on the receptor subunits [16,17]. Subsequent amplification of tyrosine phosphorylation occurs through activation of Syk kinase, which phosphorylates another substrate, linker for activation of T cells (LAT) [17,18]. LAT phosphorylation activates Phospholipase  $\gamma$ 1 (PLC $\gamma$ 1), leading to the cleavage of phosphatidylinositol into inositol trisphosphate (IP3) and diacylglycerol (DAG) [20,21]. DAG activates Protein Kinase C (PKC) and inositol triphosphate (IP3), which in turn releases calcium from the endoplasmic reticulum (ER) into the cytoplasm. This free calcium is utilized in the final stages of degranulation for the direct fusion of granules into plasma membrane. For degranulation and extracellular release of MC granules, fusion between granule membrane and plasma membrane must occur. V-soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (v-SNARE/VAMP8) on the granule membrane and t-SNARE (SNAP-23) on the plasma membrane form stable trans-SNARE complexes to guide secretory vesicles to the plasma membrane [22,23]. Additionally, multiple accessory proteins are required to regulate cognate fusion processes and to assist in the formation of various protein complexes. As in the early signaling events that occur around the

receptor, tyrosine phosphorylation is also important in regulating the final exocytic events. NSF, an ATPase and vesicle-fusion regulator, is one accessory protein of interest [24,25]. It is believed that timely dephosphorylation of tyrosine phosphorylated NSF helps to assemble and disassemble the SNARE fusion complexes, regulating granule size and degranulation events. While many of these signaling substrates may be involved in the MC degranulation responses to antibody-coated bacteria or type I fimbriated enterobacteria, only a portion of the substrates may be involved in the cytokine response to LPS, for which mediator release is not accompanied by degranulation.



**Figure 3. Schematic of the signaling pathway associated with the Fcε receptor on mast cells.**

Top panel of image shows that antigen binding to the Fcε receptor induces the phosphorylation of Syk, amplifying signaling to induce degranulation and cytokine and lipid mediator release. Middle panel of image shows the brief signaling events during IgE receptor-mediated MC degranulation. Bottom panel of image shows the signaling events involved in secretory vesicle fusion during degranulation. PTP-  
MEG2 (protein tyrosine phosphatase megakaryocyte 2) dephosphorylates the vesicle-fusion regulator NSF (N-ethylmaleimide-sensitive factor), which is a prerequisite to induce granule fusion to the plasma membrane.

### **1.1.5 Physiological Consequences of MC Degranulation.**

Although MCs produce antimicrobial agents that directly reduce the microbial burden in the body, for the most part the antimicrobial role of MCs appears to involve the mobilization of other immune cells to combat infection. The MC response to microbial challenge is typically biphasic. First, rapid degranulation facilitates the release of pre-formed inflammatory mediators, including TNF- $\alpha$ , proteases, and histamine, that initiate the early recruitment of immune cells to sites of infection [26]. This initial response is followed by *de novo* synthesis and secretion of various immune mediators several hours later. This biphasic response permits MCs not only to initiate but to sustain critical immune responses for prolonged periods of time. Because MCs are found in close proximity to the vasculature, many MC-derived mediators readily traffic into the bloodstream, initiate blood vessel dilation, and promote the extravasation of various immune cells [27,28]. The functional importance of MCs has been best studied following bacterial infections. The protective role of MCs and MC-derived TNF was first demonstrated nearly two decades ago against *Klebsiella pneumoniae* infection [29] and against polymicrobial intra-abdominal sepsis [30]. Upon contact with bacteria, MCs release TNF- $\alpha$ , which initiates the early recruitment of neutrophils to clear the pathogen.

MCs have been shown to play similar protective roles during *Pseudomonas aeruginosa* infection of the mouse peritoneum [31], *E. coli* infection of the peritoneum and urinary tract [32], *Citrobacter rodentium* [33] and *Helicobacter felis* [34] infections of the gastrointestinal tract, and *Haemophilus influenzae* infection of the ear [35]. MCs are equally effective against several Gram-positive bacteria, including *Streptococcus pyogenes*, *Mycoplasma pulmonis* [36], *Mycoplasma pneumonia* and *Listeria monocytogenes* [37]. Table 1 shows various pathogenic



bacteria that infect humans and summarizes the protective effects of MCs exerted against these pathogens.

**Table1. Diverse physiological reactions of MCs to various pathogenic bacteria**

Pathogenic bacteria are grouped by Gram-stain reaction. Each bacterium is categorized by corresponding MC mediators and their effects on the immune system. ND: not determined.

Gram Stain Reaction	Pathogenic Bacteria	MC mediators	Physiological Consequences	References
Gram-Negative	<i>Klebsiella pneumoniae</i>	TNF- $\alpha$ , IL-6	Recruitment of neutrophils	[29], [38]
	<i>Escherichia coli</i>	TNF- $\alpha$	Recruitment of neutrophils / DCs / T cells	[32], [39] [40], [30]
	<i>Escherichia coli</i>	Leukotriene	Recruitment of neutrophils	[41]
	<i>Citrobacter rodentium</i>	ND	Antibacterial activity by directly killing bacteria <i>in vitro</i>	[33]
	<i>Francisella tularensis</i>	IL-4	Alternative activation of M $\phi$ for host resistance	[42], [43]
	<i>Pseudomonas aeruginosa</i>	IL-1 $\alpha/\beta$	Recruitment of neutrophils	[44], [45]
Gram-Positive	<i>Clostridium difficile</i>	IL-8	Recruitment of neutrophils	[46], [47]
	Group A <i>streptococcus</i>	Cathelicidin	Secreting antimicrobial peptides	[48]
	<i>Mycoplasma pneumonia</i>	ND	Enhanced bacterial clearance	[36]
	<i>Listeria monocytogenes</i>	TNF- $\alpha$	Recruitment of neutrophils	[49], [50]

MCs in peripheral tissue are activated upon contact with Dengue virus, and in response they release diverse cytokines and chemokines to recruit natural killer (NK) and NK T cells to clear the virus [51-54]. It is not necessary for virus to infect MCs in order to activate them. Viral components such as the protein Fv and synthetic viral double-stranded ribonucleic acid (dsRNA) effectively activate MCs [55,56] and as a result can elicit the recruitment of CD8<sup>+</sup> T cells [57]. Although it is unclear whether MCs contribute to the clearance of human immunodeficiency virus (HIV), it appears that these cells become infected and serve as reservoirs, contributing to persistent HIV infection [58-60].

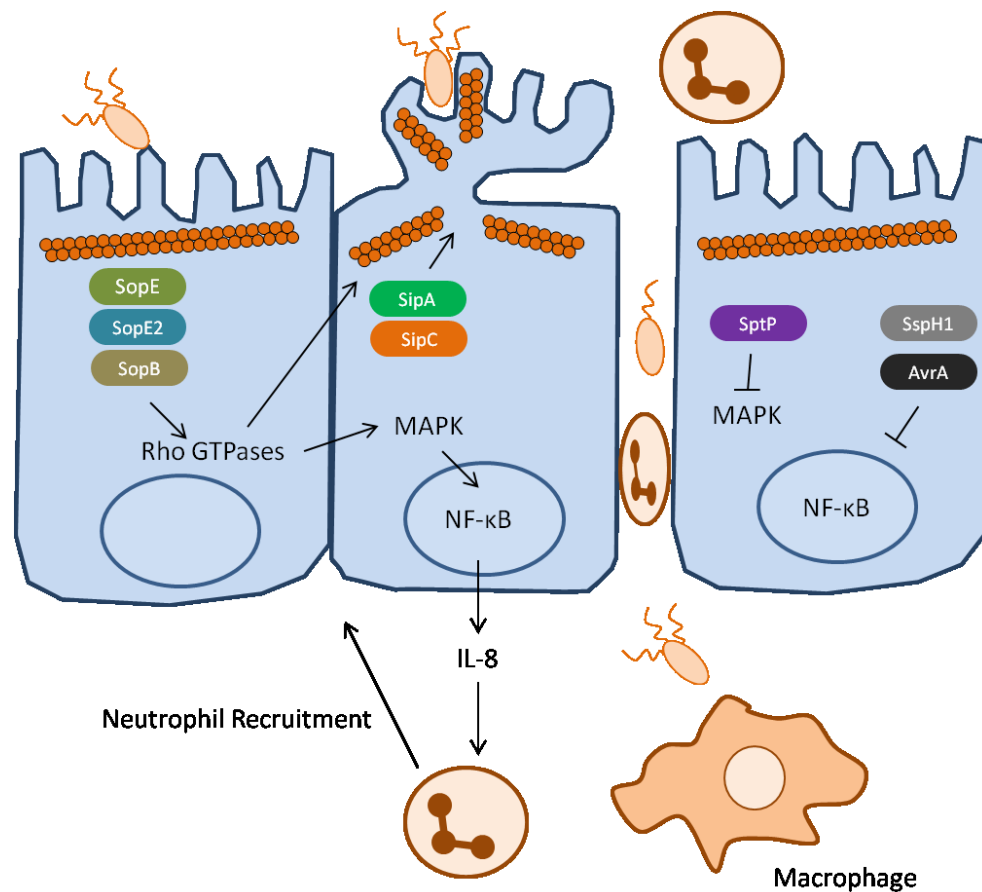
Upon contact with cell surface components of various fungi, MC *de novo* synthesize and secrete selected mediators without extensive degranulation [61]. During *Aspergillus* [62] and *C. neoformans* infections [63], MC products promote significant anti-fungal innate immunity, which mostly involves the recruitment of phagocytic cells and other immune cells. A powerful MC-mediated mechanism to reduce the burden of gut parasites such as *N. brasiliensis* [64], *Trichinella spiralis* and *Strongyloides* which is independent of immune cell recruitment, occurs via through the release of MC protease, which triggers smooth muscle contraction.

## **1.2 *Salmonella* Typhimurium**

### ***1.2.1 Salmonella Typhimurium: a Pathogenic Bacteria Involved in Food Poisoning.***

*Salmonella* Typhimurium is a highly invasive pathogen and a leading cause of foodborne illness [65]. The global burden of nontyphoidal *Salmonella* in terms of human health is as high as 93.8 million cases, but because many mild cases are not diagnosed, the number is likely much higher [66]. *S. Typhimurium* virulence has been associated with the ability to evade and

suppress the host immune system. Some of the earliest studies investigating the pathogenesis of *S. Typhimurium* highlighted its remarkable capacity to invade and persist intracellularly within gut epithelial cells and neighboring macrophages (Mφs) [67], where it can replicate while avoiding immune cells and antimicrobial agents. Invasion and intracellular persistence are mediated by a variety of effector proteins encoded in *S. Typhimurium* pathogenicity islands 1 and 2 (SPI-1 and SPI-2); the majority of these are exported out of the bacterial cell by the well-characterized type III secretion system (T3SS) [68] (Figure 4).



**Figure 4. *Salmonella* spp. utilizes the type III secretion system to achieve successful invasion and intracellular survival**

*Salmonella* secrete virulence effector proteins through the type III secretion system (T3SS), which is encoded in the *Salmonella* pathogenicity island 1 (SPI1), into the cytoplasm of host cells. Some virulence proteins (SipA, SipB, SipC, SopE and SopB) induce the rearrangement of the actin cytoskeleton, leading to bacterial uptake. Other virulence proteins (SopE, SopE2, and SopB) can activate the mitogen-activated protein kinase (MAPK) signaling pathway, which promotes pro-inflammatory cytokine secretion. Additionally, SopB destabilizes tight junctions by stimulating Cl<sup>-</sup> secretion to permit basolateral migration of neutrophils. Interestingly, along with the proinflammatory response during *Salmonella* infection, AvrA inhibits NF-κB mediated activation of the proinflammatory response. Down-regulation of the inflammatory response also occurs via SspH1. SptP fulfills dual functions by turning off MAPK signaling and reversing cytoskeleton rearrangement.

### ***1.2.2 Salmonella Typhimurium can Impede the Development of Adaptive Immune Responses.***

More recently, *S. Typhimurium* was found to directly suppress host adaptive immune responses by impeding the actions of specific immune cells; for example, *S. Typhimurium* induces antigen-presenting cells to adopt distinct migratory paths [69-71] and restricts T cell proliferation and activation to limited regions of the body following infection [72,73]. Other studies have pointed to a more global mechanism for the suppression of adaptive immune responses that involves targeting the draining lymph node, which is the epicenter of the adaptive immune response [74]. *S. Typhimurium* has been shown to target and disrupt the architecture of lymph nodes by altering homeostatic chemokine gradients, resulting in aberrant immune cell trafficking and an ineffective memory response to the pathogen.

### ***1.2.3 Salmonella Typhimurium Evades Recognition by Immune Surveillance Cells.***

The surface modification of pathogenic bacteria is a popular way to evade recognition by host surveillance cells, such as alteration of the carbohydrate capsule by *Haemophilus influenza* or *Neisseria meningitides*. Similarly, *S. Typhimurium* avoids the more immediate and non-specific host innate immune response. *S. Typhimurium* is capable of modifying lipid A expression on its surface; this is controlled by the PhoP/Q regulon. Regarding TLR4-mediated activation of the proinflammatory response, this altered surface lipid A expression is 100-fold less active than with the non-modified one [75,76].

### ***1.2.4 Salmonella Typhimurium May Also Modulate the Innate Immune Response.***

An increasing number of studies have shown that *S. Typhimurium* evasion of recognition by host immune cells cannot explain the ability of *S. Typhimurium* to rapidly proliferate, as other

bacterial components such as flagella are readily recognized by the host PRR repertoire. The lack of an adequate innate immune response to control *S. Typhimurium* growth and spread suggests a more profound bacteria-mediated mechanism to delay or completely suppress non-specific host responses.

To effectively achieve successful infection, *S. Typhimurium* has evolved virulence effector proteins to regulate host innate immune response. In particular, anti-inflammatory activity is beneficial to permit invading *S. Typhimurium* to achieve persistent infection. Although *S. Typhimurium* is known for inducing pro-inflammatory reactions in the host, recent reports suggest a role in suppressing pro-inflammatory responses in host as well. Several independent studies have reported that AvrA, acetyltransferase, inhibits the pro-inflammatory response and pro-apoptotic activity by modifying mitogen-activated protein kinase kinase, which leads to the inactivation of the c-Jun N-terminal kinase (JNK) signaling pathway [77,78]. These observations were made in intestinal epithelial cells and Mφs. In Mφs, AvrA was found to efficiently block the JNK signaling pathway and delay the death of Mφ, permitting the bacteria to establish an intracellular niche [79].

#### ***1.2.5 S. Typhimurium Survives Within Phagosomes to Establish Persistent Infections.***

After *S. Typhimurium* invades epithelial or phagocytic cells, it survives and replicates successfully in endocytic vesicles within the host cell. Infected *Salmonella* is detected inside *Salmonella*-containing vesicles (SCVs). Inside these SCVs, *S. Typhimurium* avoid the fusion of degradative vesicles by redirecting the vesicles and thus establish a *Salmonella*-favorable environment near ER without further maturation of phagosome. This establishment of *Salmonella*-favorable niche is accomplished via virulence effector proteins located in the SPI2

locus. These effector proteins prevent trafficking of NADPH oxidase- and intrinsic oxidase synthase (iNOS)-containing vesicles to SCVs [80-83]. Therefore, the failures of killing *S. Typhimurium* by resident Mφs and DCs at the submucosa lead to drain into mesenteric lymph nodes which connect bloodstream. Phagocytes harboring *S. Typhimurium* enter the blood stream and transport the bacteria into liver and spleen, eventually causing systemic infections [84]. The inability of phagocytes to eradicate *Salmonella* in phagosomes permits *Salmonella* to survive and disseminate systemically.

## **2. *Salmonella* Typhimurium Impedes Innate Immunity with a Mast Cell-Suppressing Tyrosine Phosphatase, SptP**

### **2.1 Introduction**

An important component of the innate immune response to bacterial pathogens is the MC, a morphologically distinct type of immune cell with specialized secretory functions that is preferentially located in close proximity to the epithelium of the gastrointestinal tract and other mucosal surfaces. Given their strategic location at potential sites of pathogen entry, MCs are among the first immune cells to perceive and react to microbial penetration of the epithelial barrier [3,4]. There is now a broad consensus that MCs are pivotal in initiating early innate immune responses to invading pathogens. Studies investigating Gram-positive and Gram-negative bacteria as well as viruses and fungi [26,85] have revealed that MCs promote the early clearance of pathogens.

However, to date, *S. Typhimurium* has proven to be an exception to this paradigm. Although some studies have indicated that MCs appear to contribute to *S. Typhimurium* clearance, the adoptive transfer of cultured MCs into MC-deficient mice does not significantly alter *S. Typhimurium* infection [86]. Furthermore, the presence of MCs during severe *S. Typhimurium* infection even appears harmful [87].

Given the inconclusive role of MCs during *S. Typhimurium* infection, here we sought to more closely examine interactions between MCs and *S. Typhimurium* *in vitro* and *in vivo*. We discovered that *S. Typhimurium* possessed a remarkable capacity to inhibit the ability of MCs to mount a degranulation response to powerful stimuli such as ionomycin and IgE-mediated antigen recognition and that this inhibition was attributable to SptP, a *Salmonella* protein tyrosine phosphatase secreted via the *S. Typhimurium* T3SS. Interestingly, SptP is structurally



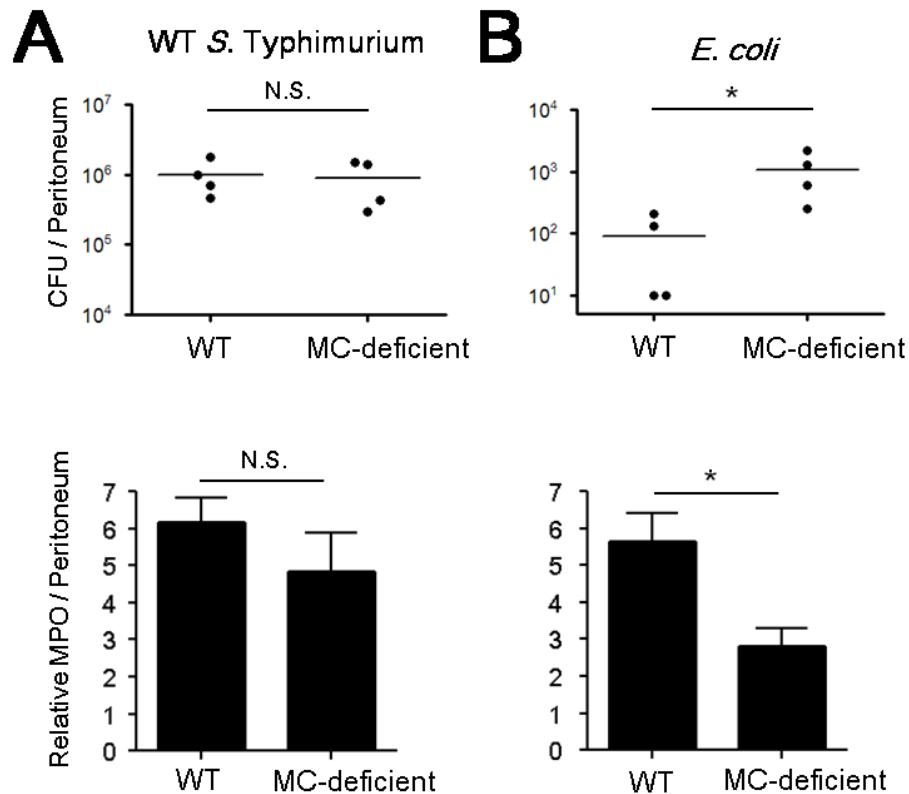
analogous to several tyrosine phosphatases present in MCs and mediators found in other pathogens, such as YopH of *Yersinia pestis*. We determined that SptP appeared to dephosphorylate at least two proteins that are critical for MC degranulation. Our studies reveal the distinct ability of *S. Typhimurium* to inactivate a key modulator of host innate immunity and thereby facilitate stealthy infection.

## 2.2 Results

### ***2.2.1 Failure of local MCs to degranulate and rapidly recruit neutrophils following *S. Typhimurium* infection***

MCs possess an apparent inability to evoke protective responses in mice following *S. Typhimurium* infection. To address this failure, we hypothesized that *S. Typhimurium* is able to inactivate MCs. In order to test this notion, we injected late log phase *S. Typhimurium* SL1433 into the peritoneal cavity of WT and MC-deficient  $\text{Kit}^{\text{W-sh}}/\text{Kit}^{\text{W-sh}}$  (MC-deficient) mice. We chose the peritoneum for the following reasons: (i) it is a self-contained body site where bacterial numbers and neutrophil influx can be simultaneously and conveniently assessed, (ii) bacteria can directly interact with MCs, and (iii) MCs can be readily visualized in the peritoneal fluid in order to determine activation status by evaluating cell morphology. Interestingly, we observed no significant difference in the bacterial burdens of peritoneal lavage from WT and MC-deficient mice upon infection with *S. Typhimurium* (Figure 5A, top). Consistent with this finding, myeloperoxidase (MPO) assays with lavage fluid revealed no significant difference in neutrophil influx between the two groups of mice (Figure 5A, bottom). In contrast, when *E. coli* J96 was intraperitoneally injected into the two mouse strains, a significant difference in bacterial clearance was observed between WT and MC-deficient mice (Figure 5B, top). This observation correlated with an increased neutrophil influx in WT mice compared to MC-deficient mice

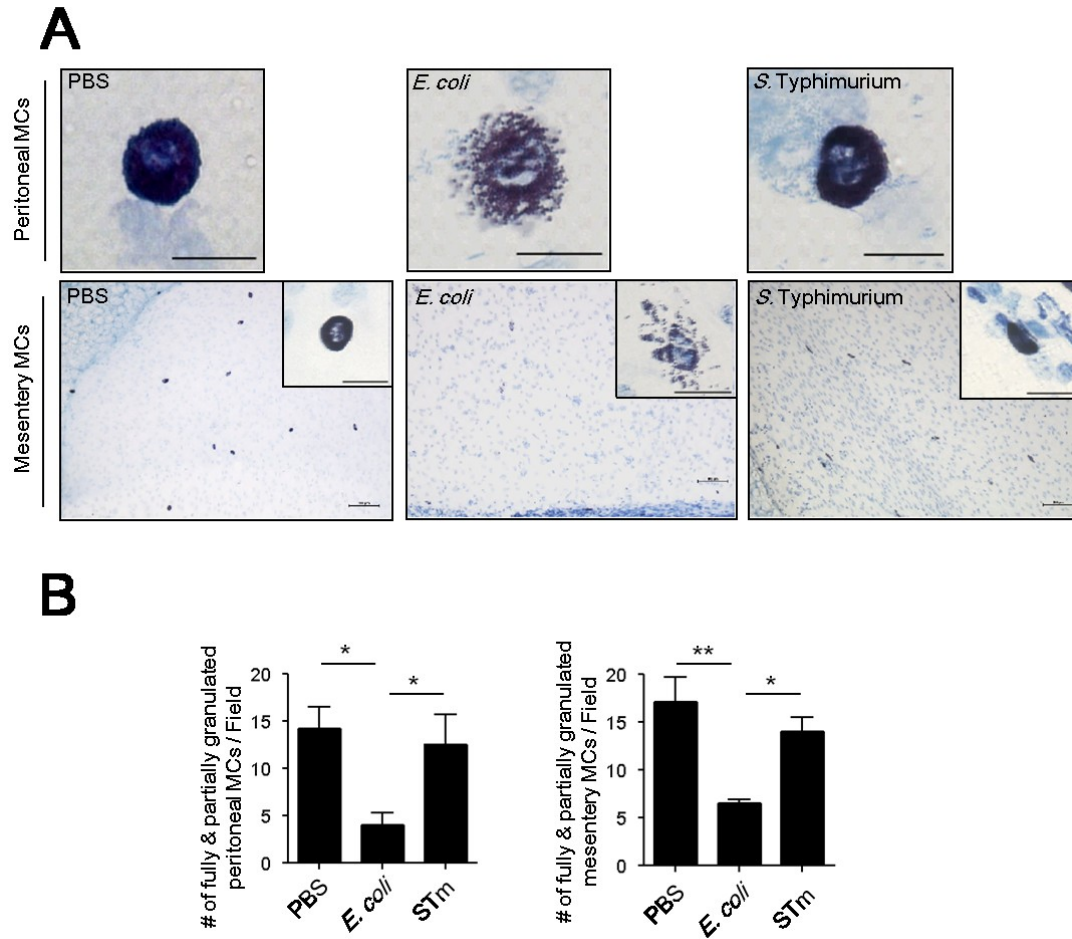
(Figure 5B, bottom). The lack of an appreciable difference in bacterial load between WT and MC-deficient mice is consistent with previous reports that found no clear protective function for MCs during *S. Typhimurium* infection [87].



**Figure 5. *S. Typhimurium* fails to elicit neutrophil recruitment and bacterial clearance *in vivo*.**

(A, B, top) Residual bacterial counts (CFUs) in the peritoneal cavity of wild-type (WT) or MC-deficient mice 24 h following intraperitoneal (i.p.) infection with  $5 \times 10^5$  CFU *S. Typhimurium* or  $1 \times 10^7$  CFU *E. coli*. (A, B, bottom) Myeloperoxidase assay with peritoneal lavage fluid from WT or MC-deficient mice 5 h post-infection with  $1 \times 10^7$  CFU *S. Typhimurium* or *E. coli* J96. Mean  $\pm$  SEM, \* $p < 0.05$ , N.S., not significant.

To determine whether our observations could be related to differential MC activation upon contact with different pathogens, we investigated the morphology of MCs in the peritoneal fluid (Figure 6A, top) and mesentery (bottom) of both groups of mice. For comparative purposes, we also examined MCs from PBS-injected mice. MCs from mice challenged with *E. coli* J96 were not readily visible with toluidine blue staining, as they were extensively degranulated with an observable spray of isolated granules around each MC (Figure 6A, middle). In contrast, MCs from *S. Typhimurium*-challenged mice were readily detectable and fully granulated (Figure 6A, right) and morphologically resembled MCs from controls (Figure 6A, left). Quantification of fully and partially granulated MCs at both of these sites is provided in Figure 6B. These observations collectively suggest that, unlike their vigorous response to *E. coli* J96, MCs appear incapable of evoking a degranulation response to *S. Typhimurium*. Consequently, limited neutrophil responses and corresponding bacterial clearance were observed in *S. Typhimurium*-infected mice.

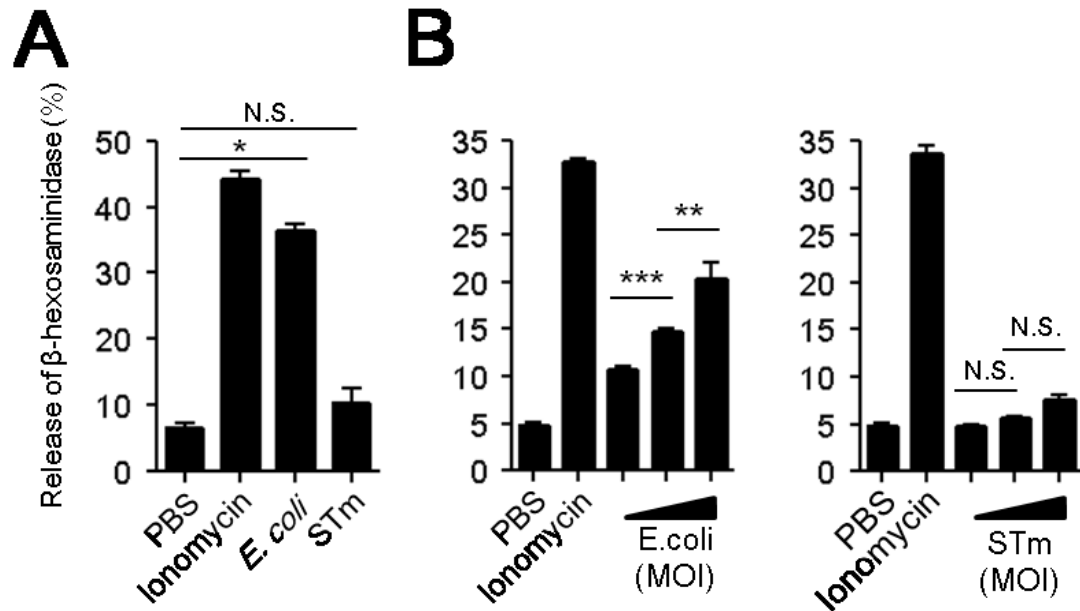


**Figure 6. *S. Typhimurium* fails to elicit MC activation *in vivo*.**

(A) Morphologic appearance of MCs in the peritoneum (top) and mesentery (bottom) of WT mice 3 h after i.p. infection with  $1 \times 10^8$  CFU *S. Typhimurium* or *E. coli* J96. Insets represent 60X magnification of MCs in the mesentery. Images represent 3 independent experiments. Top panels and inset scale: 20  $\mu$ m. Bottom panels scale: 100  $\mu$ m. n=4. (B) Granulated MC numbers in peritoneal lavages (left) or mesentery whole mounts (right) were quantified by counting the number of partially and fully granulated MCs/field (n=3-5; 5 random chosen fields). Wholly degranulated MCs could not be detected. Mean  $\pm$  SEM, \*p<0.05, \*\*P<0.01, N.S., not significant.

### 2.2.2 *S. Typhimurium* Inhibited Degranulation of Murine MCs

To more closely investigate the limited MC degranulation response to *S. Typhimurium*, we utilized the MC model cell line RBL-2H3 in standard *in vitro*  $\beta$ -hexosaminidase release assays to assess MC degranulation activity following exposure to *S. Typhimurium*, *E. coli* J96, or ionomycin, a potent MC secretagogue that works by triggering intracellular calcium flux [16]. Whereas ionomycin and *E. coli* J96 evoked significant responses, no degranulation was observed in response to *S. Typhimurium* or to PBS (Figure 7A). Compared to the dose-dependent MC degranulation observed with *E. coli* J96 (Figure 7B, left), the degranulation response to *S. Typhimurium* was minimal (Figure 7B, right).

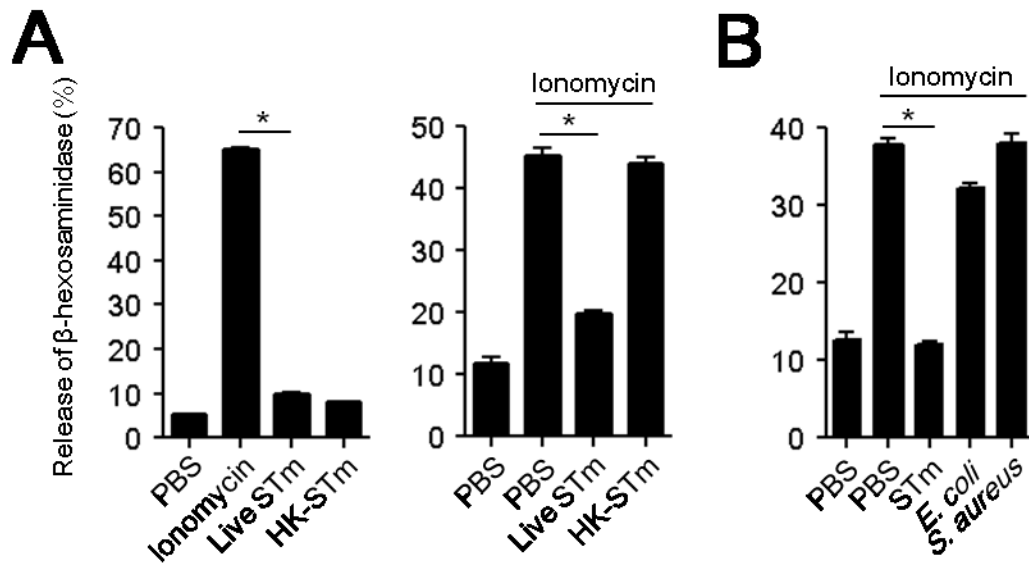


**Figure 7. *S. Typhimurium* inhibited MC degranulation, but *E. coli* activated it.**

(A, B) *In vitro*  $\beta$ -hexosaminidase release assays with RBL cells after 1 h exposure to *S. Typhimurium* (STm) or *E. coli* J96. (B) Effect of bacterial multiplicity of infection (MOIs) (10:1, 100:1, 1000:1) on MC degranulation. Mean  $\pm$  SEM, \*p < 0.001, \*\*p < 0.01, \*\*\*p < 0.05, N.S., not significant.

### **2.2.3 *S. Typhimurium* Actively Suppresses Degranulation of Murine MCs**

The limited MC degranulation to *S. Typhimurium* could potentially be attributable to the ability of *S. Typhimurium* to actively block MC degranulation or to the inability of MCs to evoke a response to *S. Typhimurium*. To test the latter possibility, we exposed MCs to live and heat-killed *S. Typhimurium* and observed that MCs failed to degranulate in response to either stimulus, indicating that the MCs were inherently unresponsive to *S. Typhimurium* (Figure 8A, left). Next, to investigate whether *S. Typhimurium* also possesses the ability to inhibit MC degranulation, we pre-treated MCs with live or heat-killed *S. Typhimurium* and then exposed the cells to the potent secretagogue ionomycin. We found that in contrast to killed *S. Typhimurium*, live *S. Typhimurium* was able to block subsequent MC responses to ionomycin (Figure 8A, right), indicating that *S. Typhimurium* has the innate capacity to actively block MC degranulation. To assess the specificity of this activity, we pre-treated MCs with live *E. coli* strain CI5 or the Gram-positive pathogen *Staphylococcus aureus* and found that neither of these bacteria were able to inhibit MC degranulation in response to ionomycin (Figure 8B).



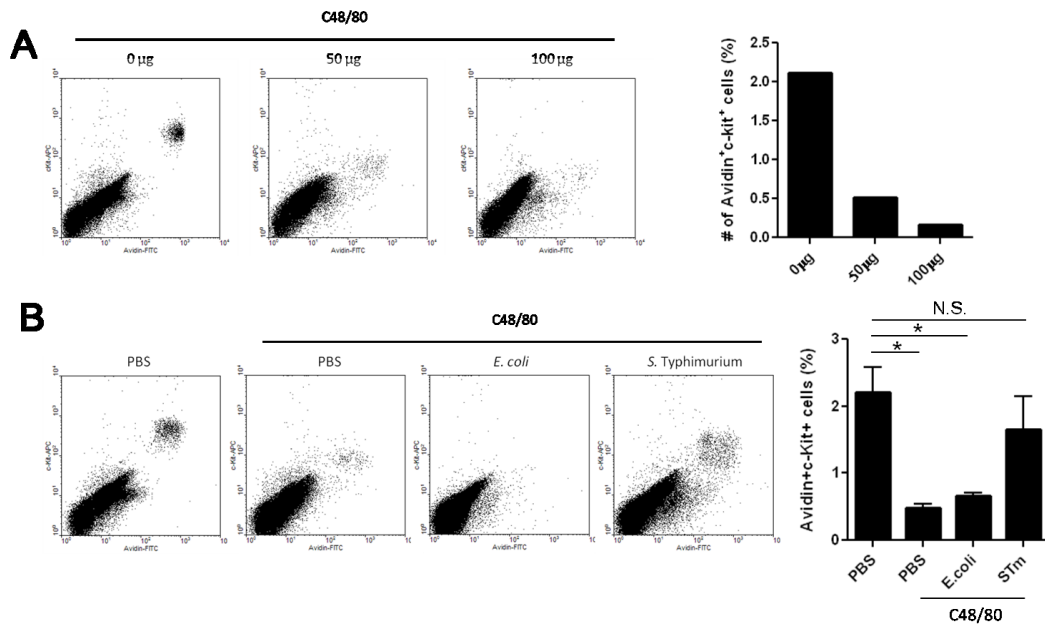
**Figure 8. *S. Typhimurium* pretreatment actively suppresses MC degranulation in response to MC secretagogues.**

(A, left)  $\beta$ -hexosaminidase release from RBLs following 1 h exposure to live or heat-treated (60°C, 1 h) *Salmonella Typhimurium* (STm). (A, right and B) RBL degranulation to ionomycin after 30 min pretreatment with (A, right) live or heat-killed (HK)-STm, and to (B) various bacteria. Mean  $\pm$  SEM, \* $p < 0.001$ .

#### **2.2.4 *S. Typhimurium* Actively Suppresses Degranulation of Peritoneal MCs *in vivo*.**

In addition to *in vitro* experiments, we tested whether *S. Typhimurium* could also block the MC degranulation response to MC activators *in vivo*. Before we started the experiment with *S. Typhimurium*, we tested whether MC degranulation could be measured *in vivo*. To induce MC degranulation, we injected intraperitoneal (i.p.) Compound 48/80 (C48/80), a MC-specific activator, and performed double staining with an anti-c-kit antibody and avidin. As Figure 9A shows, a population of c-Kit<sup>+</sup>Avidin<sup>+</sup> stained MCs were clearly visible in negative control mice, but this double-positive population dramatically decreased when C48/80 was injected. Based on

this *in vivo* model system, we first injected mice i.p. with *S. Typhimurium* or *E. coli* J96, and then injected the peritoneum with the MC-specific activator C48/80. The populations of c-Kit<sup>+</sup>Avidin<sup>+</sup> stained MCs from mice administered C48/80 alone or C48/80 + *E. coli* J96 were depleted, while a significant portion of the MC population in mice administered C48/80 + *S. Typhimurium* was still present in the peritoneum (Figure 9B).



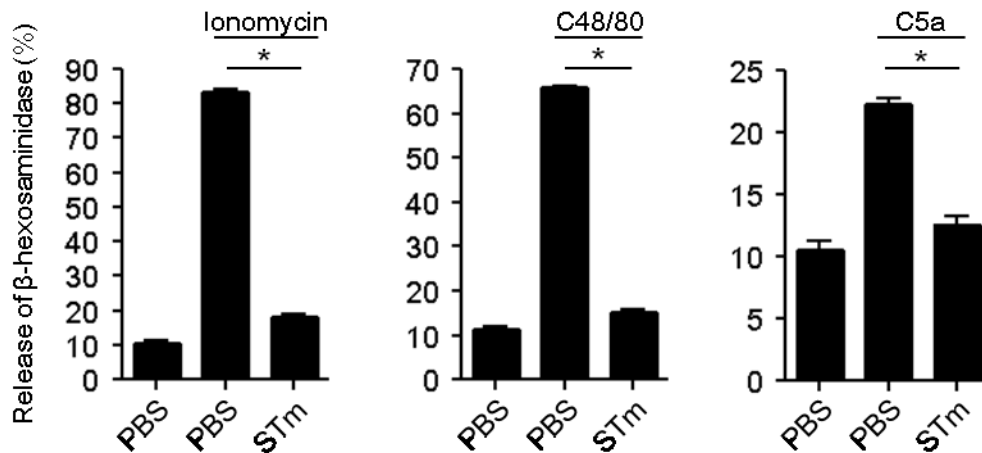
**Figure 9. *S. Typhimurium* suppresses peritoneal MCs *in vivo*.**

(A) Dose-dependent i.p. injection was performed with C48/80 (50 ug or 100 ug). (B)  $5 \times 10^8$  CFU of *S. Typhimurium* was injected i.p.. After 30 min, C48/80 (60 ug) was injected i.p. to activate MCs. (A and B) After 30 min, the peritoneal fluid was stained with an anti-c-kit antibody and avidin for flow cytometry. Bar graph indicated the percent population of avidin<sup>+</sup>c-kit<sup>+</sup> cells. Mean  $\pm$  SD, \* $p < 0.01$ .  $n = 2-5$  mice. N.S., not significant.



### 2.2.5 *S. Typhimurium* Actively Suppresses the Degranulation of Human MCs

As our previous observations were made in a rodent cell line and mice *in vivo*, we sought to confirm them in the LAD2 human MC cell line. Pre-treatment of human MCs with *S. Typhimurium* not only blocked subsequent responses to ionomycin but also to other known secretagogues, C48/80 and complement fragment C5a (Figure 10). This suggests that *S. Typhimurium* possesses both the ability to circumvent MC activation and also the capacity to suppress the MC degranulation response to activators utilizing distinct signaling pathways, suggesting multipronged inhibitory activity.

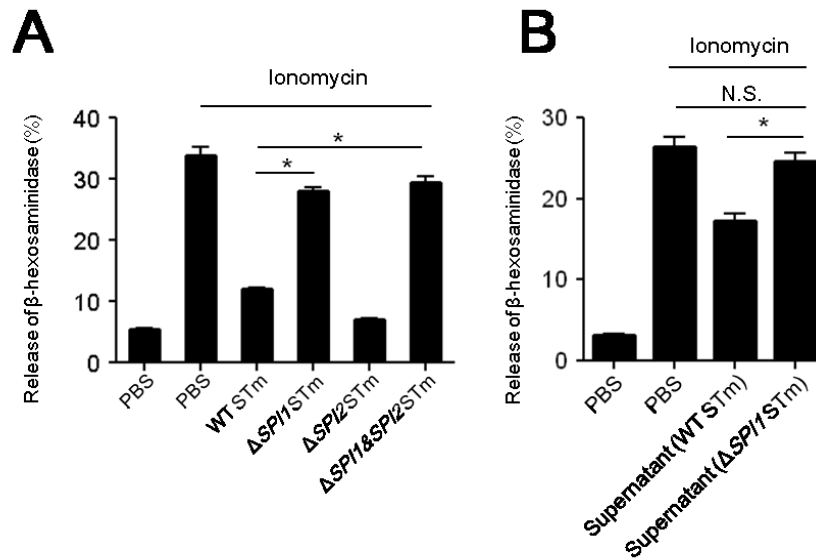


**Figure 10. *S. Typhimurium* pretreatment suppresses human MC degranulation to MC secretagogues.**

Human mast cell (LAD2) degranulation *in vitro* was analyzed with a  $\beta$ -hexosaminidase release assay in response to ionomycin (left), C48/80 (middle), or C5a (right) after 30 min pretreatment with live STm or PBS (control). Mean  $\pm$  SEM, \* $p < 0.001$ .

### ***2.2.6 MC's Suppression is Dependent on SPI-1 T3SS and a Secreted Factor Inactivates MC Degranulation***

Virulence factors associated with *S. Typhimurium* are typically encoded by genes located in one or more pathogenicity islands. Therefore, we examined *S. Typhimurium* null mutants for the two best characterized pathogenicity islands, SPI1 and SPI2, as well as a double mutant (SPI1&SP2) for their ability to block MC degranulation. In contrast to the SPI2 mutant ( $\Delta$ SPI2), we found that the SPI1 mutant ( $\Delta$ SPI1) as well as the double SPI1&SPI2 mutant ( $\Delta$ SPI1&2) failed to block ionomycin-induced degranulation (Figure 11A). The SPI1 T3SS translocates multiple effectors directly into host cells, many of which activate or impede specific cellular functions [67]. In addition to whole bacterial infection, we tested whether *Salmonella* culture supernatant retained the ability to suppress MCs (Figure 11B). The results indicated partial inhibition by the WT *Salmonella* culture supernatant compared to the  $\Delta$ SPI1 mutant supernatant, suggesting that SPI1-dependent effector molecules from *S. Typhimurium* are secreted into culture media and are responsible for MC suppression.



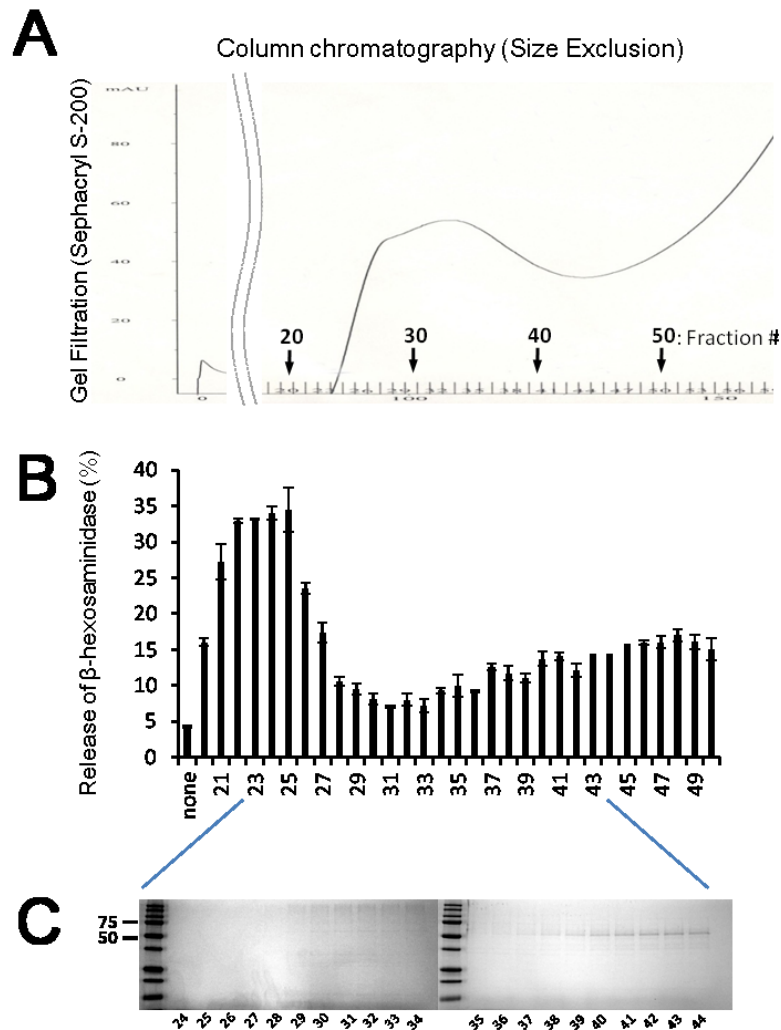
**Figure 11. MC suppression is dependent on SPI-1 T3SS and its secreted factor.**

(A) MC degranulation to ionomycin after 30 min pretreatment with WT,  $\Delta SPI1$  mutant,  $\Delta SPI2$  or  $\Delta SPI1 \& \Delta SPI2$  STm strains and (B) MC degranulation to ionomycin after 2 h pretreatment with culture supernatant from WT or  $\Delta SPI1$  mutant strain, were measured by performing a  $\beta$ -hexosaminidase release assay. Mean  $\pm$  SEM, \* $p < 0.001$ .

### **2.2.7 Effector Proteins of ~50-75 kDa in Size Retain the Most Significant MC Suppressive Activity.**

So far, we observed that heat-killed *S. Typhimurium* and  $\Delta SPI1$  *S. Typhimurium* fail to suppress MCs. *S. Typhimurium* translocates bacterial virulence effector proteins through SPI-1 T3SS and at least 20 effector proteins had been identified. At this point, we hypothesized that effector molecules having capacity to suppress MC are *Salmonella* secreted proteins. As we observed that MC suppressive effector proteins are secreted into *Salmonella* culture supernatant, we utilized column chromatography (Figure 12A), which separates these effector proteins based on protein size, to further identify these factors. If a collected fraction from the

elution of column chromatography were to demonstrate MC suppressive activity, we could narrow down the candidate factors based on size. Figure 12B shows that fraction numbers 29 through 33, which corresponded to molecular weights from 50 to 70 kDa (Figure 12C), exerted the most significant suppressive effects on MC degranulation.

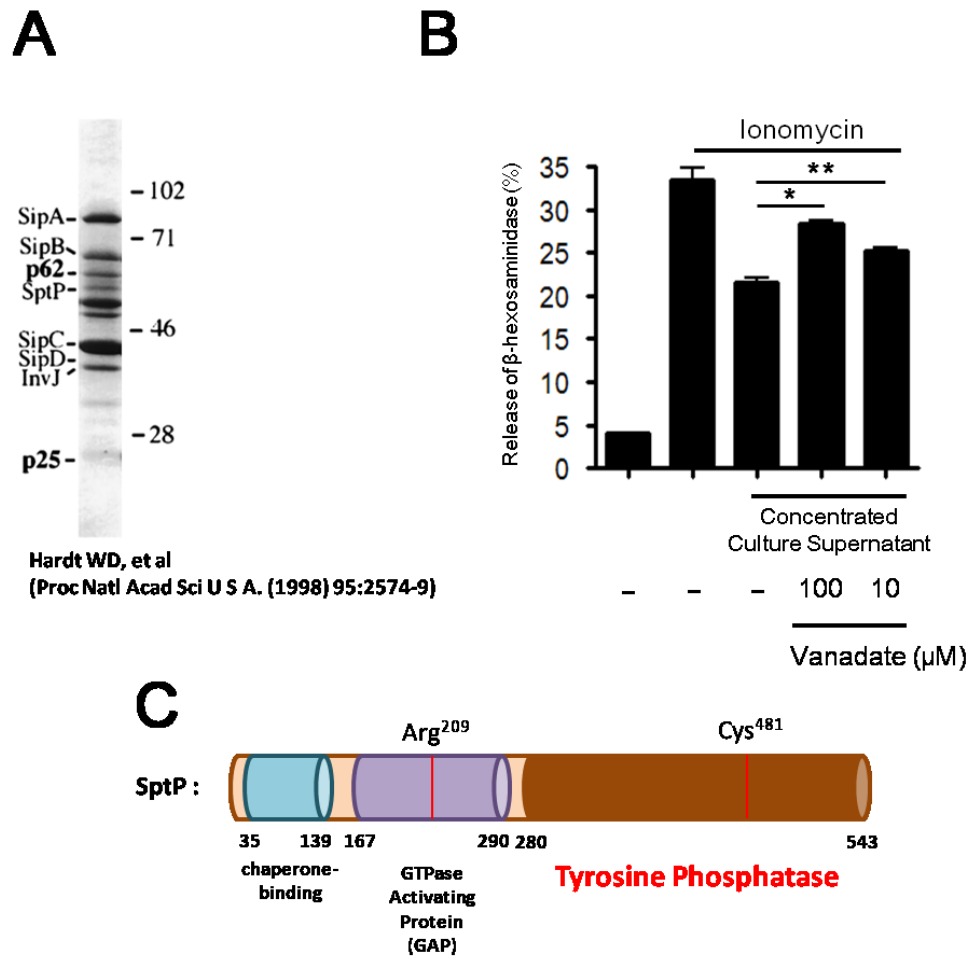


**Figure 12. Fractions 24-44 harboring ~50-75 kDa proteins demonstrate potent MC suppressive activity.**

(A) The culture media supernatant from *S. Typhimurium* was separated by column chromatography (packed with Sephacryl S-200). Eluted fractions were collected and (B) used to treat RBL cells. After 1h, the degranulation response to ionomycin was measured with a  $\beta$ -hexosaminidase release assay. (C) Fractions 24 to 44 were Coomassie stained.

A previous study done by the Galán group showed that *S. Typhimurium* secretes SPI1 effector proteins into culture media, and five effector proteins have been identified [1] (Figure 13A). Among them, two proteins are identified as ~50-70 kDa in size. When we connected these two results, the possible candidates for MC suppressive activity were *Salmonella* invasion protein B (SipB) and *Salmonella* protein tyrosine phosphatase (SptP). SipB is one of the components of the translocon complex which is assembled on the host membrane and is required for proper secretion from T3SS [88,89]. SptP retains protein tyrosine phosphatase activity to mediate host-cell recovery after bacterial invasion [90,91].

As we noticed in Figure 3, MC degranulation requires signaling cascades mediated by a tyrosine phosphorylation at each signaling molecules [16]. To inhibit overwhelming activation signals, MCs express endogenous tyrosine phosphatases near the plasma membrane to stop the tyrosine phosphorylation signaling pathway [92]. Intuitively, we hypothesized that protein tyrosine phosphatase activity from SptP is a potential candidate for MC suppression. Therefore, we tested whether *Salmonella* culture supernatant exhibits tyrosine phosphatase activity, which can interfere with tyrosine phosphorylation signaling in MCs. To inhibit tyrosine phosphatase activity in *Salmonella* culture supernatant, sodium orthovanadate was pre-incubated with MCs before treatment with ionomycin. The MC suppressive activity of *Salmonella* culture supernatant was reduced significantly by pre-incubation with the tyrosine phosphatase inhibitor (Figure 13B). Therefore, this data is consistent with the MC suppressor being SptP (Figure 13C), a known SPI1 effector with distinct phosphatase and GTPase activities [90].

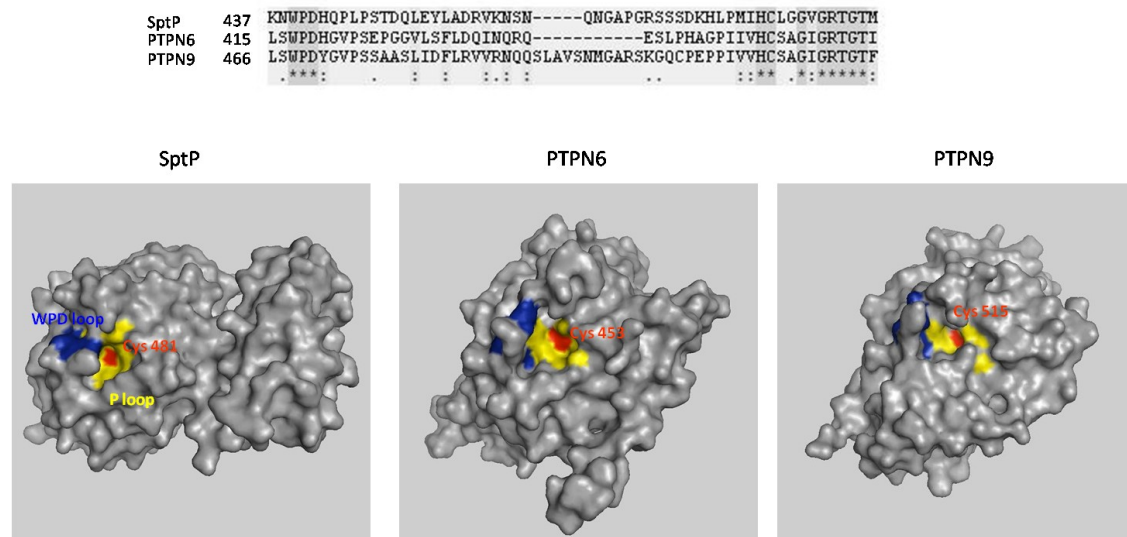


**Figure 13. Tyrosine phosphatase activity can inhibit MC degranulation.**

(A) Culture supernatant effector proteins of WT *S. Typhimurium* were separated by SDS-PAGE and stained with Coomassie blue. This data is adopted and modified from Hardt WD *et al.*[1]. (B) Culture supernatant from WT *Salmonella* was pretreated with sodium orthovanadate (100  $\mu$ M or 10  $\mu$ M) for 15 min. MC degranulation in response to ionomycin was measured by performing a  $\beta$ -hexosaminidase release assay after 2 h incubation with pretreated culture supernatant. (C) Diagram of SptP with two functional domains: GTPase activating protein (GAP) and protein tyrosine phosphatase. Mean  $\pm$  SEM, \* $p$ <0.001, \*\* $p$ <0.05.

### 2.2.8 Comparison of MC Tyrosine Phosphatase with SptP of Salmonella.

We have noted that SptP shares significant homology with phosphatases naturally found in MCs [90,92,93]. To compare SptP in *Salmonella* to protein tyrosine phosphatase 6 (PTP6) and PTP9 in human cells including MCs, a sequence alignment and structural comparison were performed. Figure 14 shows that catalytic active site and functional motifs in SptP are structurally homologous to PTPs in human. Therefore, SptP can potentially target signaling molecules in MCs and interfere with tyrosine phosphorylation signaling in MCs.



**Figure 14. Sequence alignments of tyrosine phosphatase in *S. Typhimurium* and MCs.**

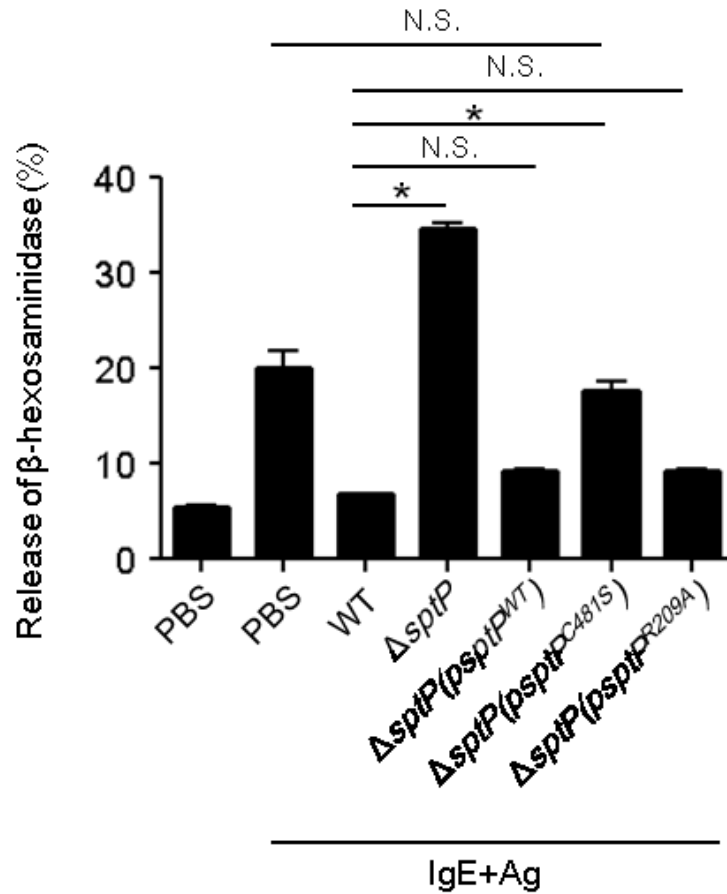
(Top) Protein tyrosine phosphatase of *S. Typhimurium* is sequentially conserved with PTP in MCs. PTPN6 and PTPN9 in MCs share the following functional and characteristic protein tyrosine phosphatase motifs with SptP in *S. Typhimurium*. Cysteine residue (red) located in the active site is highly conserved in the three proteins. This cysteine residue is a phosphotyrosine binding site in the phosphatase. The PTP signature motif (yellow), which has the “HCXAGXGR(S/T)” amino acid sequence in the catalytic domain, is also highly conserved in the three proteins. The WPD loop (blue) which phosphatases utilize to dephosphorylate the target is also conserved in the three proteins.

### **2.2.9 SptP-Mediated Suppression of IgE+ $\alpha$ IgE-induced MC degranulation.**

To investigate the role of SptP in suppressing MC activity, we first compared the ability of WT *S. Typhimurium* and an isogenic  $\Delta sptP$  mutant to prevent degranulation. Here, we used IgE+antigen as the MC stimulant because this well-characterized signaling pathway has several potential targets for phosphatase activity. We found that in the case of MCs pretreated with the  $\Delta sptP$  mutant, the degranulation response to IgE stimulation was no longer inhibited and was even higher than that seen with IgE stimulation (Figure 15). One possibility for the enhanced degranulation response of  $\Delta sptP$  treated MCs is that the GTPase-activating protein (GAP) domain of SptP also has a known ability to promote recovery of the cellular cytoskeleton after *Salmonella*-mediated invasion [91]. In the absence of a reorganized cytoskeleton, as is the case with the  $\Delta sptP$  mutant, MCs degranulate excessively following stimulation [94].

Next, we undertook a series of complementation studies to identify the relevant region in the functionally distinct domains located at either end of SptP. To distinguish between the effects of the GTPase-activating function of the amino-terminal region and the protein tyrosine phosphatase function associated with the carboxy-terminal region [90,91], we complemented  $\Delta sptP$  *S. Typhimurium* with the plasmid *psptP<sup>WT</sup>* encoding WT *sptP*, *psptP<sup>C481S</sup>* encoding *sptP* with an inactive phosphatase domain, or *psptP<sup>R209A</sup>* encoding *sptP* with an inactive GTPase domain and compared the ability of the complemented strains to suppress MC degranulation (Figure 15). Complementation with *psptP<sup>WT</sup>* or with *psptP<sup>R209A</sup>* but not with *psptP<sup>C481S</sup>* inhibited MC degranulation in comparison to the  $\Delta sptP$  mutant. These results suggest that SptP, and specifically the catalytic activity of its tyrosine phosphatase domain, mediates suppression of MC degranulation.

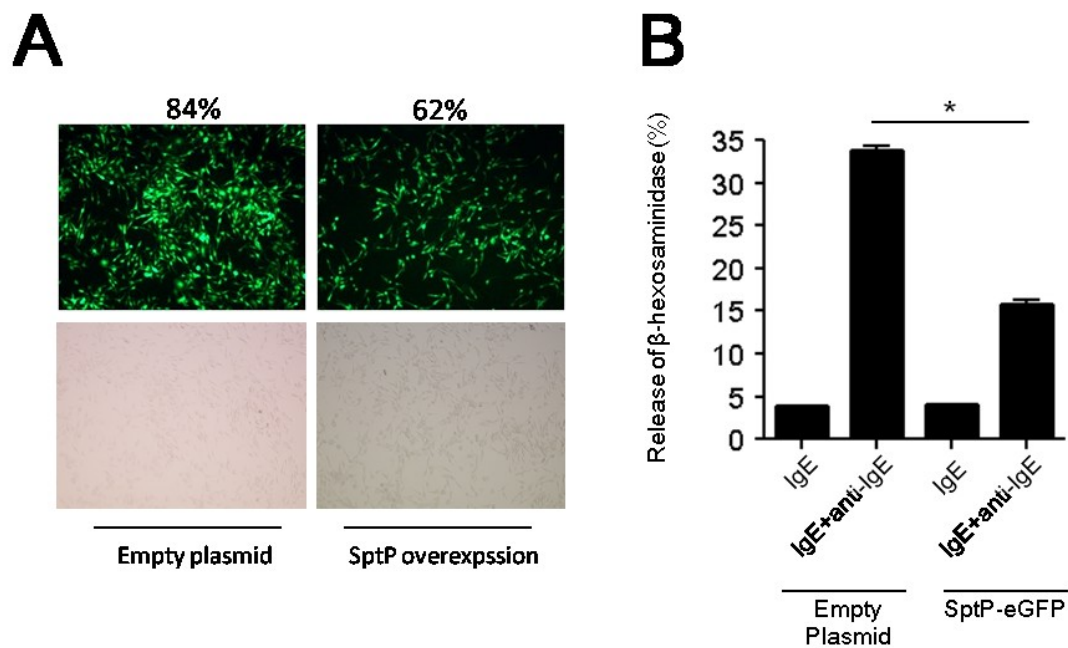




**Figure 15. The protein tyrosine phosphatase domain in SptP inhibits MC degranulation.**

Degranulation of IgE sensitized MCs in response to antigen (Ag, TNP-OVA) after 45 min pretreatment with WT,  $\Delta sptP$  or complemented  $\Delta sptP$  strains ( $\Delta sptP(psptP^{WT})$ ,  $\Delta sptP(psptP^{C481S})$ , or  $\Delta sptP(psptP^{R209A})$ ). Mean  $\pm$  SEM, \*p<0.001.

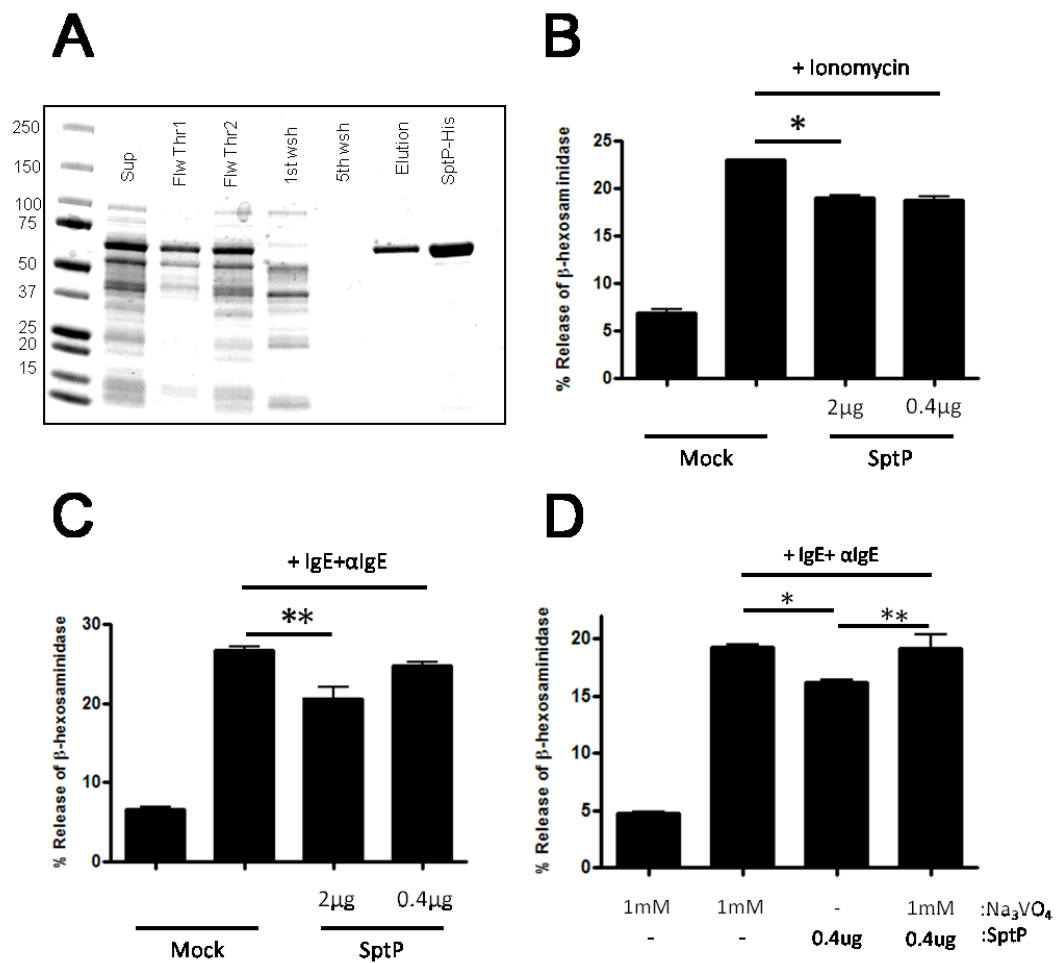
To investigate whether SptP has the inherent ability to suppress MC degranulation, we cloned and expressed SptP intracellularly in the RBL-2H3 MC cell by stably transfecting with SptP-eGFP, resulting in SptP expression in approximately 60 % of cells based on fluorescent activity (Figure 16A). We next examined the effects of SptP expression on the MC degranulation response to IgE+anti-IgE in RBL-2H3 cells. We observed that RBL-2H3 MC cells transfected with SptP-eGFP exhibited a significant (~60 %) reduction in degranulation compared to the control (Figure 16B).



**Figure 16. SptP-eGFP stable expression in MCs suppresses MC degranulation.**

(A) Expression of transduced eGFP or SptP-eGFP was assessed by green fluorescence in transfected cells (top). Corresponding bright field images are shown at the bottom. (B) IgE sensitized MCs expressing retrovirally transduced eGFP or SptP-eGFP were cross-linked by anti-IgE and analyzed with a  $\beta$ -hexosaminidase release assay.

Next we investigated whether applying exogenous recombinant SptP into the culture supernatant of MCs would block their subsequent degranulation. RBL-2H3 cells were treated with increasing concentrations of recombinant SptP purified from *E.coli* BL21 (Figure 17A) and then examined for their degranulation in response to different amounts of ionomycin (Figure 17B) or to IgE+anti IgE (Figure 17C). In both cases, a modest level of inhibition of the degranulation response was observed. To confirm that tyrosine phosphatase activity from SptP mediates suppression of MC degranulation, we pretreated recombinant SptP with orthovanadate and examined its ability to block MC degranulation responses to IgE+anti IgE (Figure 17D). However, we noticed that suppression with recombinant SptP was much less than with *S. Typhimurium* by itself. We reasoned that the modest ability of recombinant SptP to block MC degranulation may be attributable to its relative inability to permeate MC membranes as this effector protein is typically introduced by *S. Typhimurium* directly into the MC cytosol via its T3SS [68].

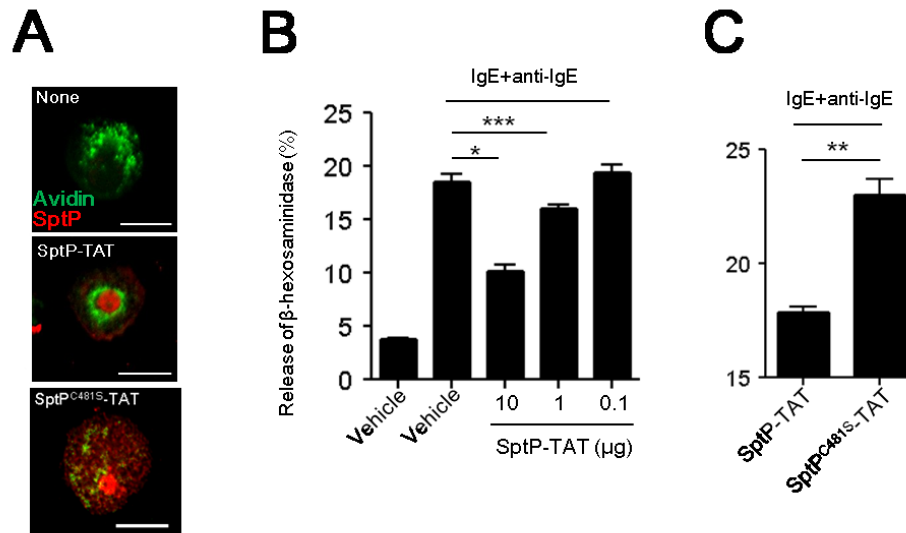


**Figure 17. Introduction of recombinant SptP into MC culture media suppresses MC degranulation.**

(A) SptP-His<sup>6</sup> was overexpressed in BL21 *E. coli* and purified with a Ni column. (B and C) Purified recombinant SptP-His<sup>6</sup> and (D) SptP-His<sup>6</sup> with sodium orthovanadate (1mM) were incubated with RBL-2H3 cells. After overnight incubation, the degranulation response to (B) ionomycin (1 $\mu$ g/ml) or (C and D) anti-IgE antibody was measured with  $\beta$ -hexosaminidase release assays. Mean  $\pm$  SEM, \*P<0.001, \*\*p<0.05.

### ***2.2.10 Transduction of SptP-TAT into MCs Significantly Suppresses MC Degranulation.***

To facilitate trafficking of SptP into the MC cytosol via non-endocytic pathways after exogenous treatment with SptP, we constructed membrane-permeant SptP by conjugating it to the short peptide RKKRRQRRR derived from the HIV TAT (transactivator of transcription) protein, which can translocate itself into the cytosol of various host cells [95]. To observe SptP-TAT penetrance into bone marrow-derived mast cells (BMMCs), we employed confocal microscopy to visualize the protein within BMMCs. We opted to employ BMMCs here to make the point that observations made using the RBL-2H3 cell line are also applicable to primary MCs. We observed that SptP-TAT readily entered BMMCs and could even be detected in the nuclei (Figure 18A, bottom). Due to its superior ability to penetrate MC membranes, we predicted that SptP-TAT would effectively block the MC degranulation response. We observed that in contrast to controls, SptP-TAT significantly inhibited IgE+anti-IgE-mediated degranulation in a dose-dependent manner (Figure 18B). We also generated a catalytically inactive form of SptP-TAT, SptP<sup>C481S</sup>-TAT, to specifically ablate tyrosine phosphatase activity, that was observed in the cytosol and nuclei (Figure 18A). Unlike SptP-TAT, recombinant SptP<sup>C481S</sup>-TAT was unable to inhibit IgE+anti-IgE-mediated BMMC degranulation (Figure 18C), confirming that SptP inhibits degranulation via its tyrosine phosphatase activity.

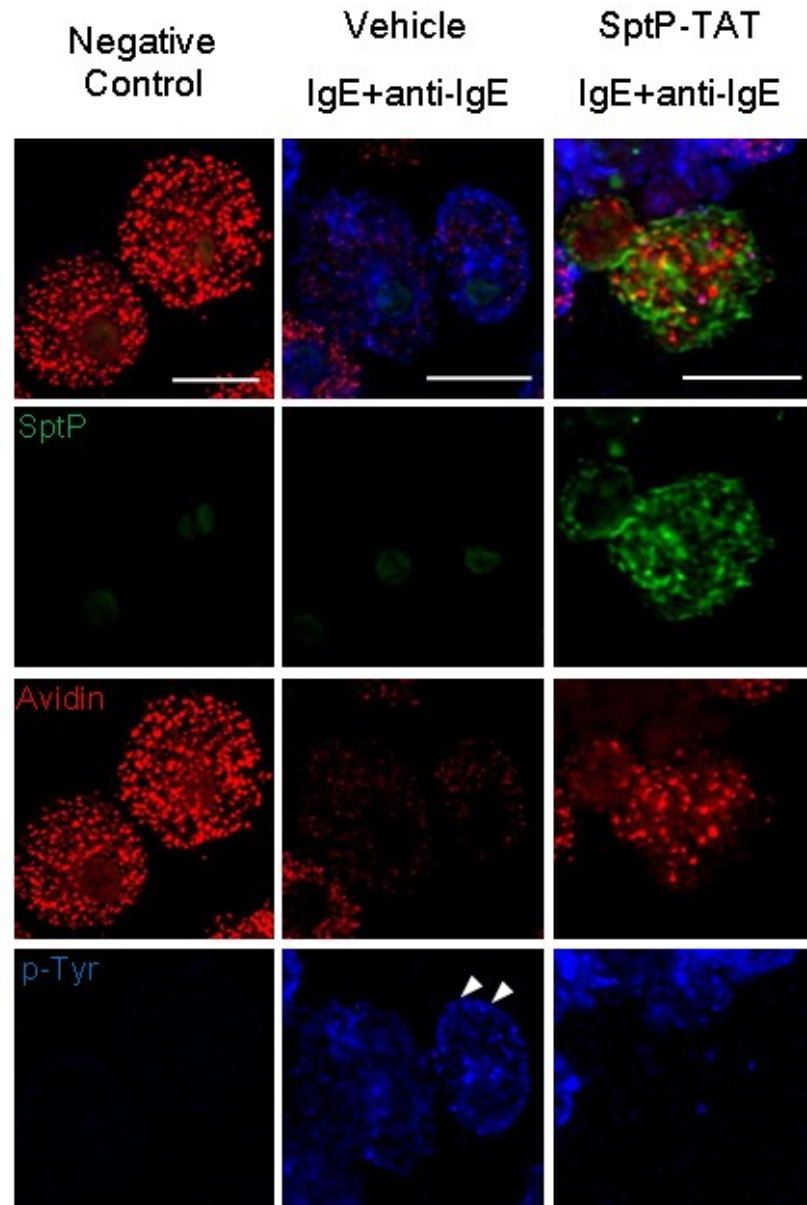


**Figure 18. Treatment with SptP-TAT suppresses IgE-anti-IgE mediated MC degranulation.**

(A) Confocal microscopy of BMMCs exposed to SptP-TAT or SptP<sup>PC481S</sup>-TAT for 4 h. MC granules were probed with avidin (green) and intracellular SptP-TAT was probed with anti-His<sub>6</sub> (red). (B and C) Degranulation of IgE-sensitized BMMCs in response to anti-IgE (B) after 4 h pretreatment with increasing amounts of SptP-TAT (10, 1, and 0.1  $\mu$ g) or (C) after 2 h pretreatment with SptP-TAT (5  $\mu$ g) or SptP<sup>PC481S</sup>-TAT (5  $\mu$ g). Scale bar is 20  $\mu$ m. Mean  $\pm$  SEM, \* $p$ <0.001, \*\* $p$ <0.01, \*\*\* $p$ <0.05

### ***2.2.11 SptP Impedes Tyrosine Phosphorylation Signaling in MCs***

Next, we sought to localize sites of tyrosine phosphorylation in MCs during degranulation and determine their activation following exposure to SptP-TAT. Confocal microscopy of BMMCs employing probes for MC granules and phosphotyrosine revealed that tyrosine phosphorylation appeared to be localized primarily to empty compartments where granules were housed prior to activation with IgE+anti-IgE (Figure 19, middle, arrowheads). This is consistent with the notion that not only is tyrosine kinase activity important in the signaling events leading to degranulation, but it is also important in the final stages of granule release [24]. We thus hypothesized that intracellular SptP could interfere with the regulation of the granule secretory pathway to block MC degranulation. In SptP-TAT-treated BMMCs (Figure 19, right), which did not degranulate following exposure to IgE+anti-IgE, no tyrosine phosphorylation was observed in the granule chambers, which is consistent with a lack of degranulation. Notably, detectable amounts of SptP were localized to the granule compartments. Thus, the absence of tyrosine phosphorylation proximal to sites of granule discharge contributes to abrogated MC degranulation in SptP-TAT-treated MCs.



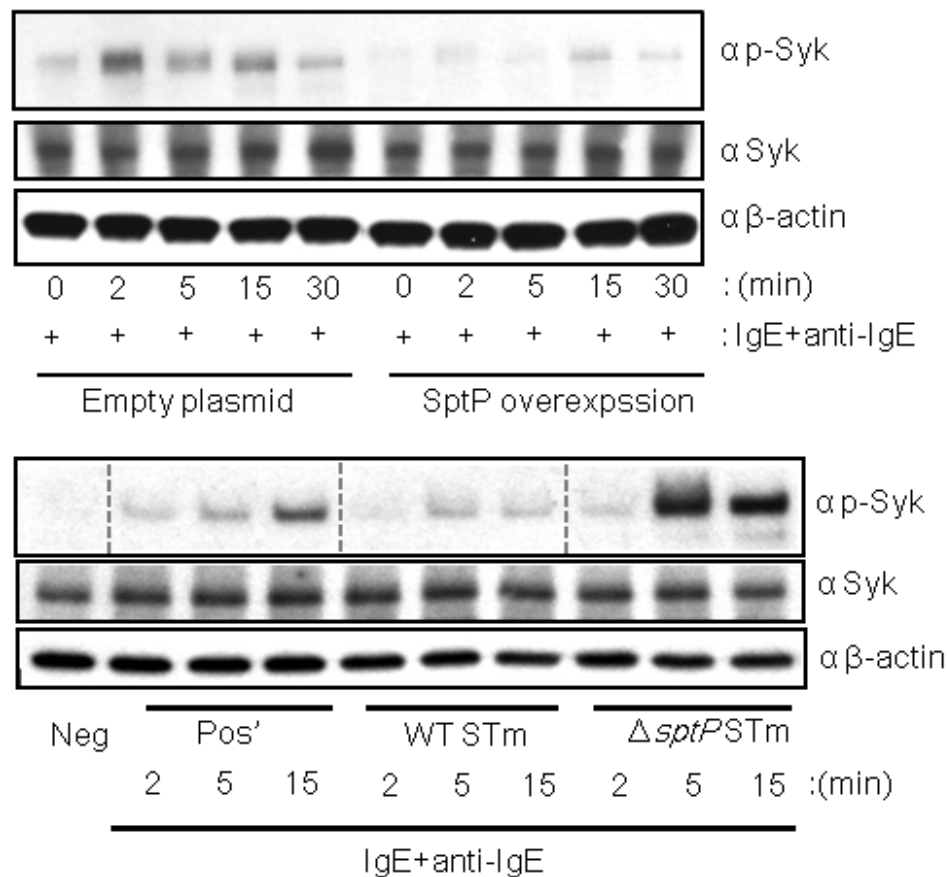
**Figure 19. SptP-TAT inhibited tyrosine phosphorylation in MC granule chambers to block MC degranulation.**

Morphological appearance of IgE sensitized BMMCs in response to anti-IgE after 2 h pretreatment with vehicle or SptP-TAT (10  $\mu$ g). MC granules: avidin (red), SptP-TAT: anti-His<sub>6</sub> (green), and intracellular tyrosine phosphorylation sites: anti-phosphotyrosine (p-Tyr) (blue) antibodies. Scale bars: 20  $\mu$ m.



### ***2.2.12 SptP Dephosphorylates Syk, an Early Signaling Substrate Following Receptor-mediated Activation of MCs.***

The SptP-TAT-associated absence of tyrosine phosphorylation in MCs led us to seek putative SptP targets in the IgE signaling pathway. Syk, a tyrosine kinase, is an important substrate in IgE-mediated MC degranulation [16,96], and thus we examined the effects of SptP on Syk activation following IgE mediated activation. SptP overexpression in transfected MCs markedly suppressed Syk phosphorylation following stimulation with IgE+anti-IgE (Figure 20, top). To further confirm the role of Syk, we also compared IgE-mediated Syk phosphorylation in MCs pre-treated with WT or  $\Delta sptP$  *S. Typhimurium*. Consistent with SptP overexpression, WT *S. Typhimurium* markedly suppressed Syk phosphorylation, whereas  $\Delta sptP$  *S. Typhimurium* failed to do so. Indeed,  $\Delta sptP$  *S. Typhimurium* appeared to enhance Syk phosphorylation, indicating that in the absence of SptP, *Salmonella* might directly activate MCs and that the signaling events involve Syk (Figure 20, bottom).



**Figure 20. Syk, one of tyrosine phosphorylation cascade, is the target of SptP.**

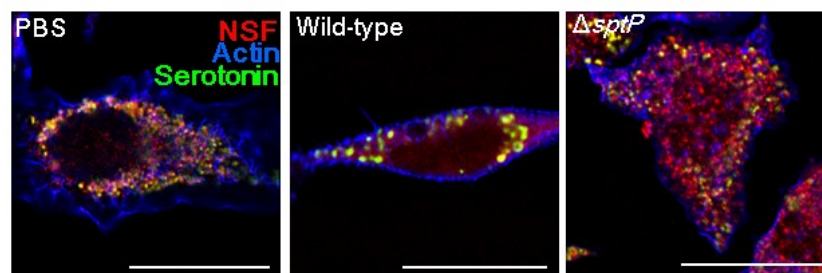
Western blots of cell preparations from IgE-sensitized RBLs that either stably expressed SptP-eGFP or control eGFP (top) or were pretreated with WT STm or  $\Delta sptP$  STm for 1 h (bottom) before activation with anti-IgE. The cell preparations from both experiments were obtained at the indicated time points after activation with anti-IgE. Western blots were probed with  $\alpha$ -p-Syk,  $\alpha$ -Syk, and  $\alpha$ - $\beta$ -actin antibodies.

### ***2.2.13 SptP Dephosphorylates NSF, a Signaling Substrate Implicated in Final Stages of Granule Release in MCs.***

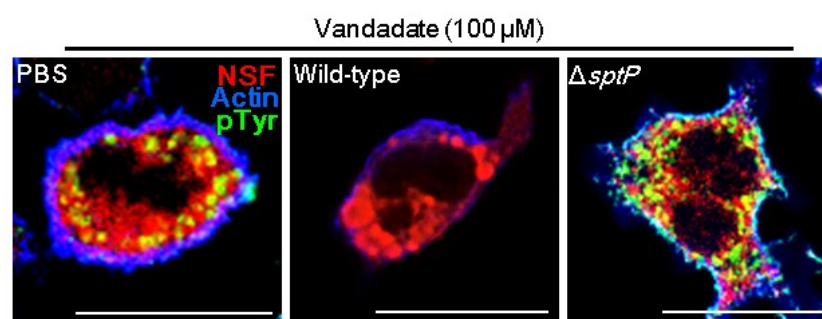
We also noticed that although granules of RBL-2H3 cells were not released following 1 h exposure WT *S. Typhimurium*, they appeared to have fused with each other, resulting in abnormally large intracellular granules (Figure 21A, middle). However, this phenotype was not observed in MCs exposed to  $\Delta sptP$  *S. Typhimurium* (Figure 21A, right). A critical determinant of MC degranulation is NSF, a vesicle fusion protein localized to the cytoplasmic side of the granule membrane [24,25]. In the steady state, NSF is typically phosphorylated which is important to prevent MC granules from spontaneous intracellular fusion [97]. Upon MC activation, NSF became dephosphorylated, promoting the fusion of granule membranes to plasma membranes and resulting in extracellular release of MC granules. We hypothesized that in WT *S. Typhimurium*-infected MCs, NSF is prematurely dephosphorylated, resulting in granule fusion without extracellular release. To validate this hypothesis, control-treated, WT, and  $\Delta sptP$  *S. Typhimurium*-infected MCs were stained for NSF. In all cases, NSF colocalized to the outer surface of MC granules (Figure 21B). Furthermore, the granules of WT *S. Typhimurium*-infected MCs appeared to be 2-3 times the size of regular granules of control MCs and  $\Delta sptP$  *S. Typhimurium*-infected MCs, suggesting intergranular fusion in the former (Figure 21B). To determine if intergranular fusion was associated with the dephosphorylation of NSF, we examined phosphorylation of NSF around MC granules and found that whereas NSF appeared phosphorylated around MC granules of PBS or  $\Delta sptP$  *S. Typhimurium*-treated MCs, this was not the case in WT *S. Typhimurium* treated MCs (Figure 21B, middle). Furthermore, when we immunoprecipitated NSF from the three groups of MCs and probed with anti-phosphotyrosine antibodies on a western blot, we observed that in contrast to the other two conditions, NSF in WT *S. Typhimurium*-infected MCs was markedly dephosphorylated (Figure 21C) providing

support for our hypothesis of premature intergranular fusion induced by *S. Typhimurium* phosphatases. That SptP acts at sites downstream of Syk can also be inferred by the fact that WT *S. Typhimurium*, but not  $\Delta sptP$  and  $\Delta sptP(psptP^{C481S})$  blocked ionomycin induced degranulation (Figure 21D), which occurs downstream of receptor-mediated MC signaling events [16].

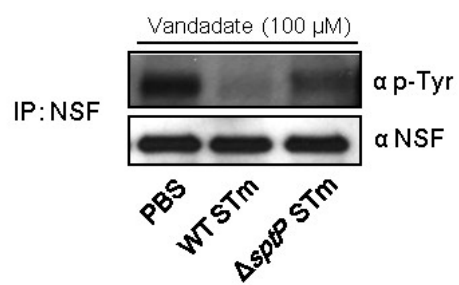
**A**



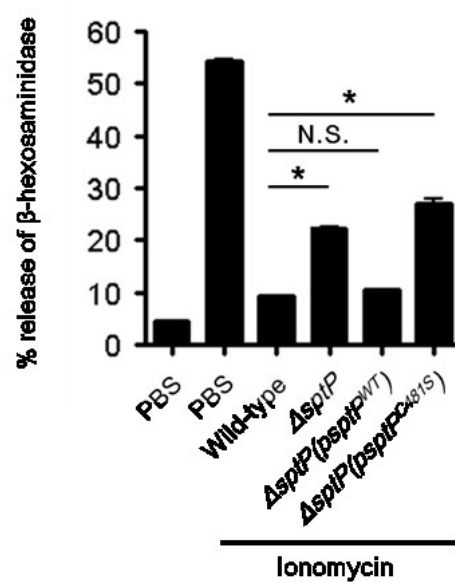
**B**



**C**



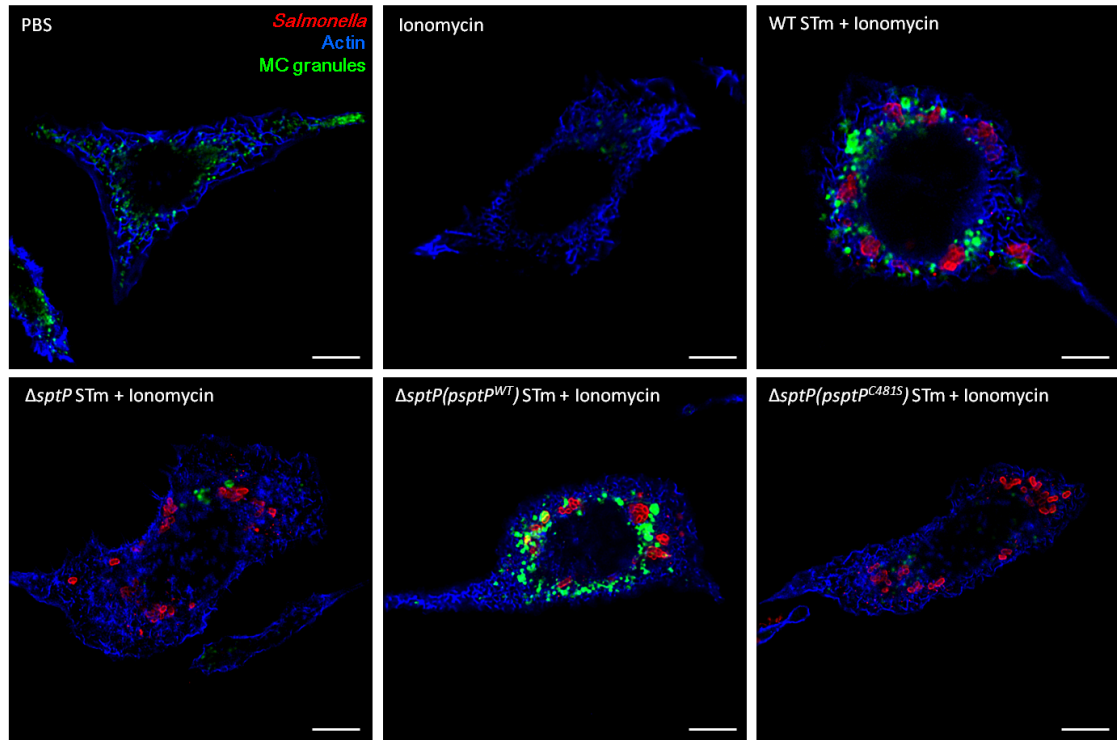
**D**



**Figure 21. Intergranular fusion induced by WT *Salmonella* through dephosphorylation of NSF.**

(A, B) Morphology of RBLs after exposure to WT or  $\Delta sptP$  STm. After 1 h treatment, (A) cells were stained with antibodies for fluorescence microscopy. MC granules: anti-serotonin (green), NSF: anti-NSF (red) antibodies and actin: phalloidin (blue). (B, C) After 1 h treatment, cells were treated with vanadate (100  $\mu$ M) for 5 min to preserve tyrosine phosphorylation and (B) were stained with antibodies for fluorescence microscopy. NSF: anti-NSF (red) and intracellular tyrosine phosphorylation: anti-pTyr (green) and actin: phalloidin (blue). (C) Lysates from RBLs were analyzed by immunoprecipitation and immunoblotting for phosphotyrosine and NSF. Scale bar is 20  $\mu$ m. (D) Degranulation responses of MCs to ionomycin after 30 min pretreatment with wild-type,  $\Delta sptP$ , or complemented  $\Delta sptP$  strains ( $\Delta sptP(psptP^{WT})$ , or  $\Delta sptP(psptP^{C481S})$ ). Mean  $\pm$  SEM, \*p<0.001

Consistent with the  $\beta$ -hexosaminidase assay results (Figure 21D), the fluorescent microscopy images showed that WT and  $\Delta sptP(psptP^{WT})$  *S. Typhimurium* retained significant amount of granules in the cytoplasm and were larger in size than those in control MCs when infected MCs were treated with ionomycin (Figure 22, top right and bottom middle). On the other hand,  $\Delta sptP$  and  $\Delta sptP(psptP^{C481S})$  failed to inhibit degranulation such that very few granules were detected in cytoplasm (Figure 22, top middle, bottom left, and bottom right), which is similar to the ionomycin alone-treated control.



**Figure 22. Morphology of RBLs after exposure to WT or  $\Delta sptP$  mutants with ionomycin.**

Morphology of RBLs after exposure to WT or  $\Delta sptP$  or  $\Delta sptP(psptP^{WT})$  or  $\Delta sptP(psptP^{C481S})$  *S. Typhimurium*. *Salmonella*: anti-*Salmonella* (red) antibody and granule: avidin (green) and actin: phalloidin (blue). Scale bar: 10  $\mu$ m.

### 2.2.14 *Yersinia* Secretes YopH to Suppress MC Degranulation.

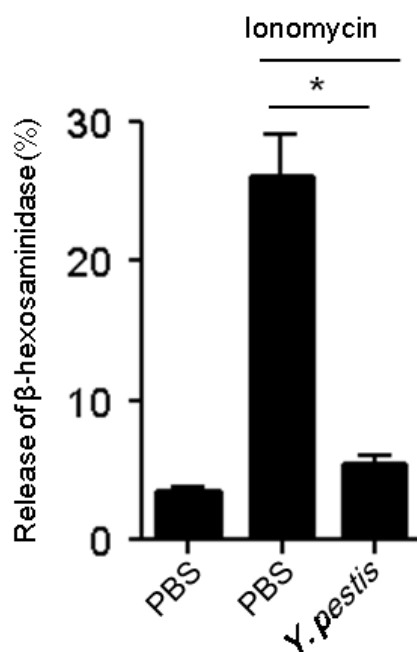
*Yersinia pestis* expresses YopH, which is structurally analogous to the tyrosine phosphatase, SptP, present in *Salmonella* Typhimurium (Table 2) [90,98]. Importantly, this enzyme is also injected into host cells via T3SS [99]. In view of the similarities in terms of the structure and delivery of these phosphatases, we speculated that MC suppression may be an evolved virulence trait shared among sophisticated pathogens such as *S. Typhimurium* and *Yersinia* and hypothesized that YopH could also suppress MC degranulation. We exposed MCs to a WT laboratory strain of *Yersinia pestis* KIM5 and examined the subsequent ability of these MCs to mount degranulation to ionomycin. *Y. pestis*-exposed MCs exhibited a significantly inhibited degranulation (Figure 23).

**Table 2. Sequence alignments of tyrosine phosphatase in *Yersinia pestis*, *Salmonella* Typhimurium, and MCs.**

PTPase of *S. Typhimurium* is sequentially conserved with PTPase in MCs. Functional homologues of *S. Typhimurium* SptP exist in *Y. pestis* and MCs. YopH in *Y. pestis* and SHP in MCs share the following characteristic PTPase motif with SptP in *S. Typhimurium*. (1) A cysteine residue located in the active site (red) is highly conserved in the three proteins. This cysteine residue is a phosphotyrosine binding site in the phosphatase. (2) The PTP signature motif, which has these “HCXAGXGR(S/T)” amino acid sequence in the catalytic domain, is also highly conserved in three proteins. (3) The WPD loop, which phosphatases utilize to dephosphorylate the target, is also conserved in the three proteins.

Sequence Alignments of Tyrosine Phosphatase in <i>Y. pestis</i> , <i>S. Typhimurium</i> , <i>MCs</i>															
YOPH_YERPS	DMYTLTIREAGQKTISVPVVHVGN	WPD	QTAVSSEVTKALASLVDQTAETKRNMYESKGSS	389											
SPTP_SALTY	DQYNMQL-SCGEKRYTIPVLHVKN	WPD	HQPLP--STDQLEYLADRVKNSNQ-----NGAP	467											
PTN6_HUMAN	RTLQVSPLDNGDLIREIWHYQYLS	WPD	HGVPS--EPGGVLSFLDQINQRQE-----	441											
	:	.	*:	:	:	.	***:	.	.	:	:	*:	:	::	
YOPH_YERPS	AVGDDSKLRPVIH	C	RAGVGRTAQLIGAMCMNDSR-----NSQLSVEDMVSQMRVQRNGIM	444											
SPTP_SALTY	GRSSDKHLPMIH	C	LGGVGRGTGTMAAALVLKD-----NPHSNLEQVRADFRDSRNNRM	520											
PTN6_HUMAN	--SLPHAGPIIVH	C	SAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMV	499											
	.	:	::**	.	*:***:	:	:	:	:	.	....	.	*	*..	:





**Figure 23. *Yersinia pestis* suppresses MC degranulation.**

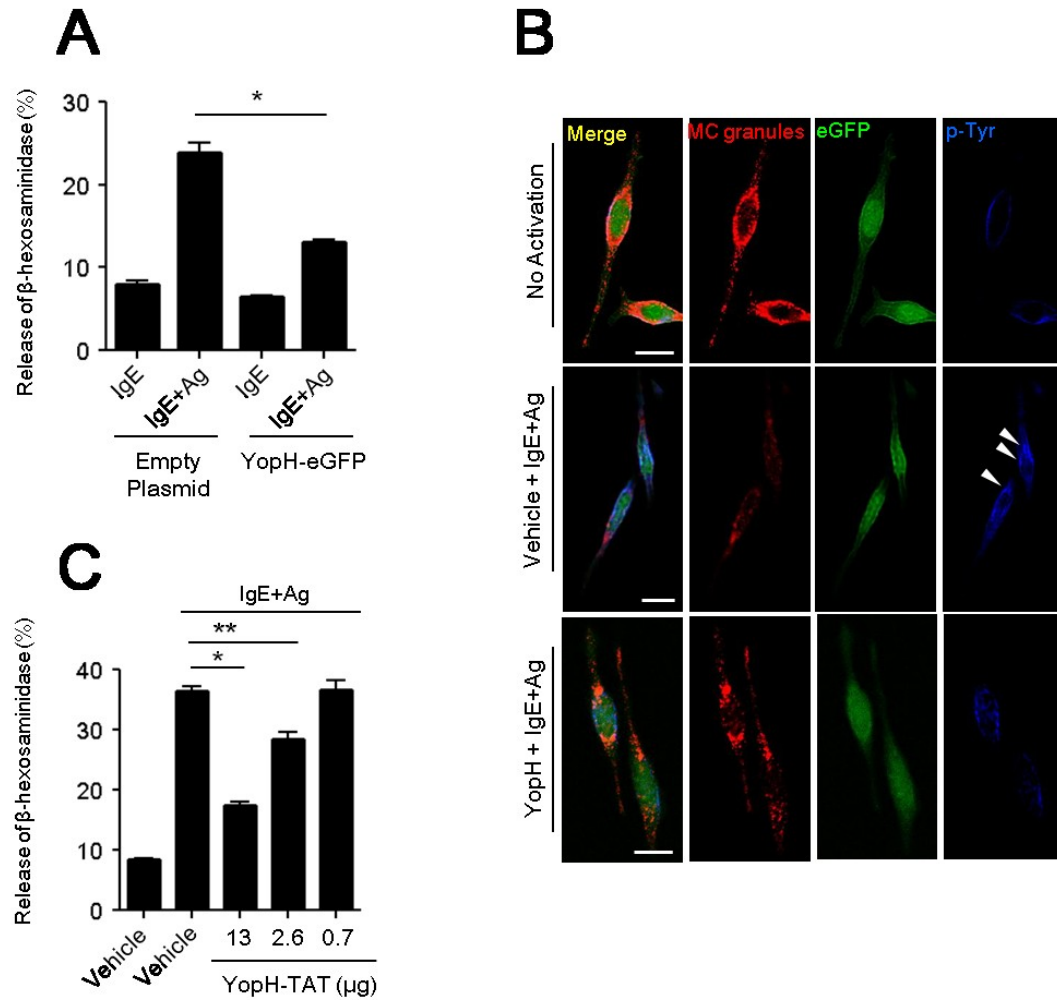
Degranulation of RBLs pretreated for 30 min with PBS or *Y. pestis* KIM5 in response to ionomycin. Mean  $\pm$  SEM, \* $p$ <0.001.

To determine whether YopH was the specific *Yersinia* factor involved in MC suppression, we cloned and expressed this tyrosine phosphatase in RBL-2H3 cells. In contrast to control eGFP-transfected cells, MCs transfected with YopH-eGFP exhibited limited degranulation in response to IgE+antigen (Figure 24A). Figure 24B demonstrates the intracellular expression of YopH, the granulation status of treated MCs, and the tyrosine phosphorylation of eGFP- and YopH-eGFP-transfected RBL-2H3 cells following exposure to IgE+antigen. In particular, MC degranulation appeared to be directly correlated with tyrosine phosphorylation in empty

granule chambers (Figure 24B, middle, arrow heads) of IgE+antigen activated control MCs but not in YopH-expressing MCs (Figure 24B).

Next, we cloned and expressed YopH as a fusion protein with TAT. To determine whether exogenous YopH-TAT would inhibit MC degranulation, we exposed IgE-sensitized MCs to increasing concentrations of YopH-TAT and examined their responses to antigen-induced activation. We observed a significant and dose-dependent suppression of the MC degranulation response to antigen (Figure 24C). Taken together, these observations indicate that YopH shares the ability of SptP to suppress degranulation and suggest that MC suppression may be an important virulence trait among host-adapted pathogens.

In this data, we observed that YopH targeted MCs to suppress innate immunity. Previous studies show that YopH can suppress adaptive immunity. The tyrosine phosphatase activity of YopH has been known to suppress the activity of lymphocytes [100]. Specifically, YopH dephosphorylates adaptor proteins to interfere with the T cell receptor signaling pathway and prevent the development of protective immunity against *Yersinia* infection [101,102]. Altogether, *Yersinia* utilizes a potent tyrosine phosphatase to inactivate diverse immune cells and down-regulate innate and adaptive immunity.

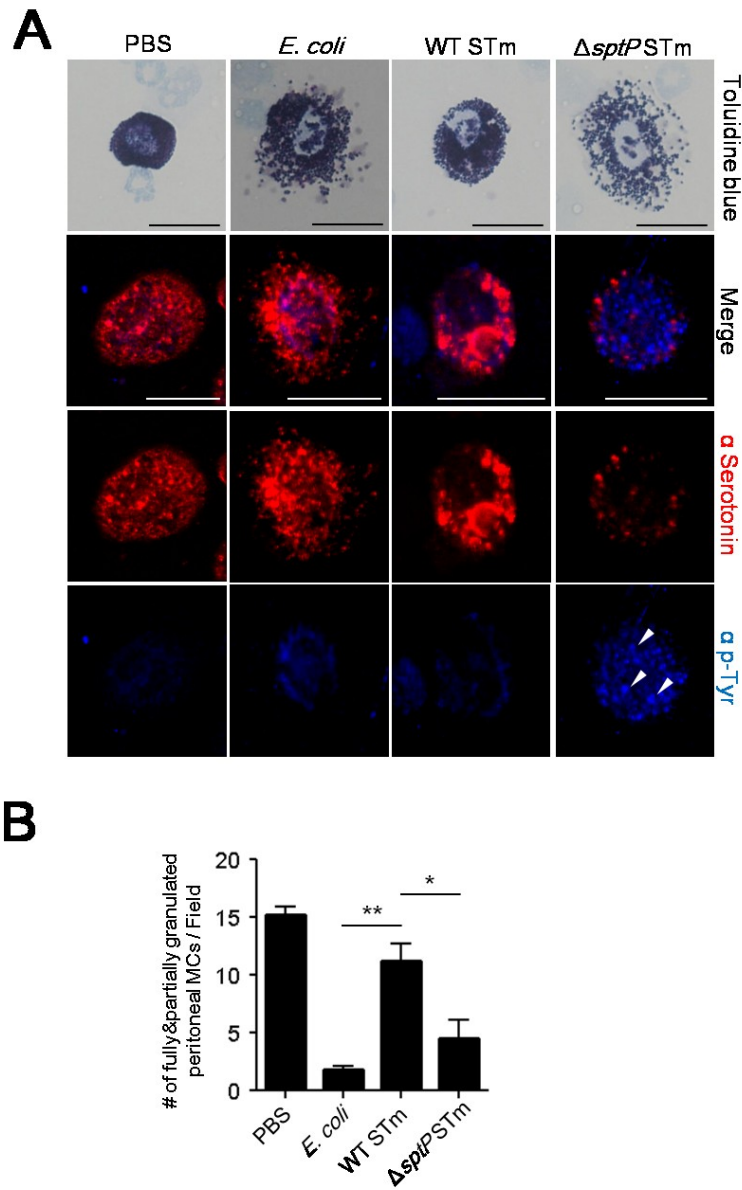


**Figure 24. *Yersinia pestis* YopH suppresses MC activation.**

(A and B) RBLs were retrovirally transduced with control eGFP or YopH-eGFP before IgE+antigen stimulation (Ag, TNP-OVA) and were assayed for degranulation (A) or subjected to confocal microscopy (B). Granules were stained with serotonin (red) and tyrosine phosphorylation was stained with an anti-p-Tyr antibody (blue). (C) Degranulation in response to IgE+Ag (TNP-OVA) RBLs following exposure to increasing concentrations of purified YopH-TAT. Scale bar: 10  $\mu$ m, mean  $\pm$  SEM, \* $p$ <0.001, \*\* $p$ <0.01.

### **2.2.15 $\Delta$ sptP *S. Typhimurium* Induces MC Degranulation**

To investigate the *in vivo* contribution of SptP to MC suppression during *S. Typhimurium* infection, we challenged the peritoneal cavities of mice with WT or  $\Delta$ sptP *S. Typhimurium* (Figure 25A) and determined that MCs isolated from the peritoneal cavities exhibited widely different phenotypes in terms of their granulation status. MCs exposed to WT *S. Typhimurium* remained fully granulated (Figure 25A, third column), but MCs exposed to  $\Delta$ sptP *S. Typhimurium* underwent extensive degranulation (Figure 25A, fourth column). This degranulation in response to the  $\Delta$ sptP mutant when quantitated was comparable to the degranulation observed with *E. coli* J96 (Figure 25B), which was included for comparative purposes. The vacant granule chambers in extensively degranulated MCs stained strongly for tyrosine phosphorylation (Figure 25A, fourth column, arrowhead), whereas granule chambers in MCs exposed to WT *S. Typhimurium* or PBS failed to exhibit tyrosine phosphorylation staining. We observed that the overall phosphotyrosine fluorescence in  $\Delta$ sptP-infected MCs was markedly higher (Figure 25A, fourth column) than *E. coli*-infected MCs. This enhanced tyrosine phosphorylation was previously observed in  $\Delta$ sptP-exposed MCs *in vitro* (data not shown), which also consistently exhibited higher Syk phosphorylation than IgE+anti-IgE positive control cells (Figure 20, bottom). This is potentially due to the fact that significantly higher degranulation was observed with  $\Delta$ sptP *S. Typhimurium* than with the positive degranulation control (Figure 15).

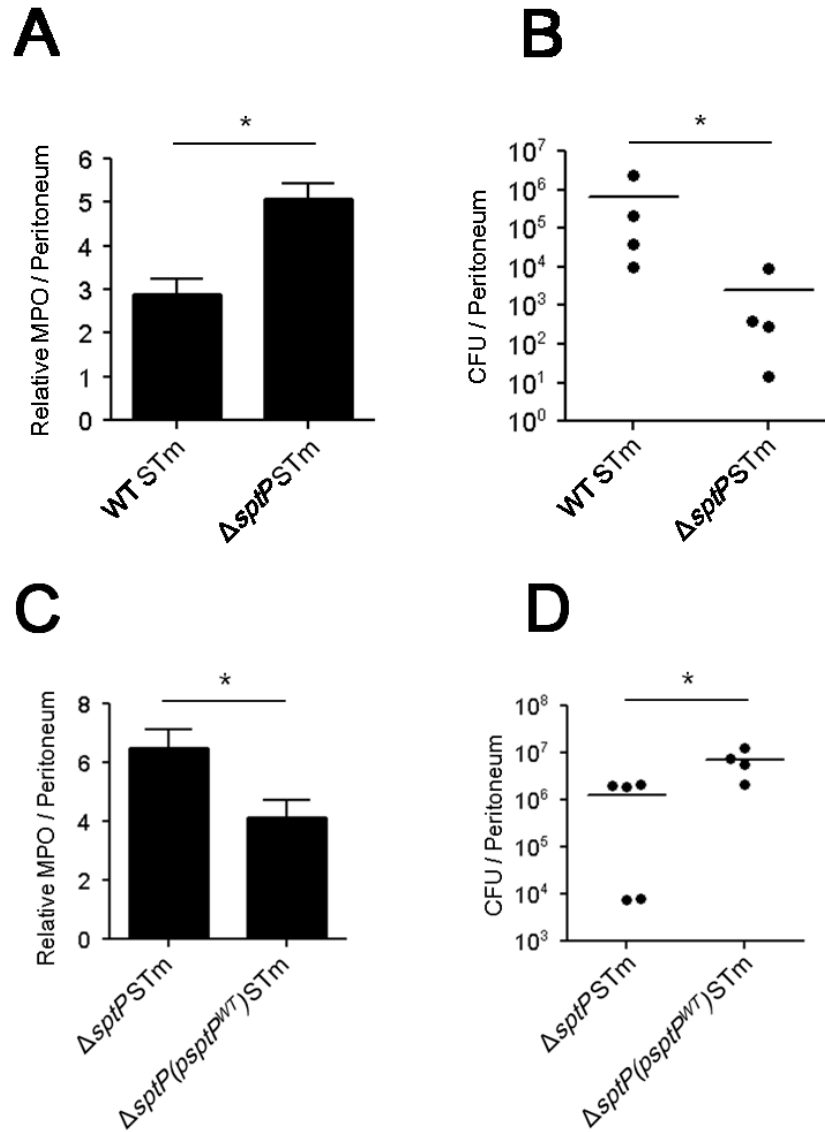


**Figure 25.  $\Delta sptP$  *Salmonella* initiated phosphotyrosine signaling and degranulation of MCs.**

(A)  $1 \times 10^8$  CFU WT STm,  $\Delta sptP$  STm, or *E. coli* J96 were injected i.p. MCs were harvested 4 h later by peritoneal lavages, cytopun, and stained with toluidine blue for bright field microscopy (top) or with  $\alpha$ -serotonin (red) for granules, and  $\alpha$ -phosphotyrosine (blue) for immunofluorescence microscopy. (B) Granulated MC numbers in peritoneal lavages of (A) were quantified by counting partially and fully granulated MCs/field (n=3-6; 5 random chosen fields). Wholly degranulated MCs could not be detected. n=4 mice, mean  $\pm$  SEM, \*p<0.05, \*\*p<0.01. Scale bar: 20  $\mu$ m.

### ***2.2.16 $\Delta sptP$ *S. Typhimurium* Triggers Neutrophil Influx and Bacterial Clearance.***

As MC degranulation has been closely associated with neutrophil recruitment and subsequent bacterial clearance, we compared neutrophil responses in the peritoneum of mice following 4 h challenge with WT or  $\Delta sptP$  *S. Typhimurium*. Predictably, we observed significantly higher neutrophil responses with  $\Delta sptP$  than with WT *S. Typhimurium* (Figure 26A), and this correlated with greater clearance of  $\Delta sptP$  compared to WT *S. Typhimurium* 24 h after bacterial challenge (Figure 26B), which was recovered with the complemented mutant strain ( $\Delta sptP(psptP^{WT})$ ) (Figure 26C, D).



**Figure 26. Enhanced neutrophil recruitment and bacterial clearance with  $\Delta sptP$  *Salmonella* infection, and decreased effects with  $\Delta sptP(psptP^{WT})$  *Salmonella*.**

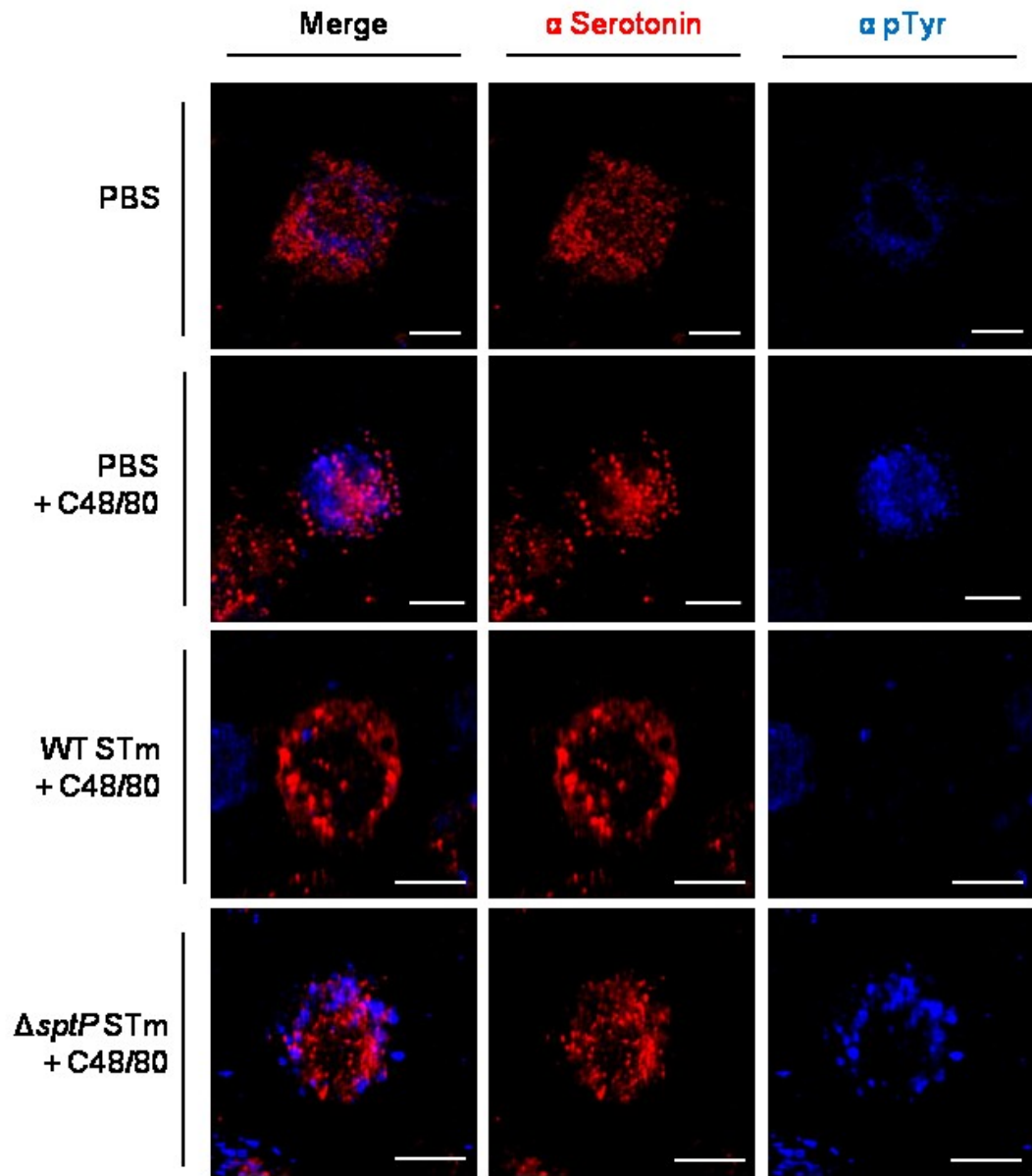
(A) Assays for neutrophil recruitment (MPO) and (B) bacterial clearance (CFUs) were performed on lavages collected 4 h and 24 h, respectively, after i.p injection of  $1 \times 10^6$  CFU WT or  $\Delta sptP$  STm; n=4 mice. (C) Assays for neutrophil recruitment (MPO) and (D) bacterial clearance (CFUs) were performed on peritoneal fluids collected 4 h and 24 h, respectively, after i.p. injection of  $1 \times 10^6$  CFU  $\Delta sptP$  or  $\Delta sptP(psptP^{WT})$  complemented mutant strain STm. n=4-5 mice, Mean  $\pm$  SEM, \*p<0.05.

### ***2.2.17 $\Delta$ sptP S. Typhimurium Fails to Suppress MC Degranulation Induced by C48/80, Resulting in Neutrophil Influx and Bacterial Clearance.***

Thus far, we observed that WT *S. Typhimurium* suppresses MC degranulation and following neutrophil recruitment and bacterial clearance was affected, but  $\Delta$ sptP *S.*

*Typhimurium* fails to do so. Next, we investigated whether *S. Typhimurium*-mediated MC suppression continues even when a potent MC activator is administered. To test this hypothesis, mice with WT *S. Typhimurium* or  $\Delta$ sptP *S. Typhimurium* i.p. infection (1h) were challenged with C48/80 i.p. (30min). As Figure 27 shows, both C48/80 alone and  $\Delta$ sptP *S. Typhimurium* + C48/80 induced heavy degranulation from peritoneal MCs (second and fourth rows, respectively). In contrast, peritoneal MCs from mice with WT *S. Typhimurium* infection failed to undergo degranulation even when they were exposed to C48/80 (third row). This suggests SptP mediated dephosphorylation is suppressing MC activation.

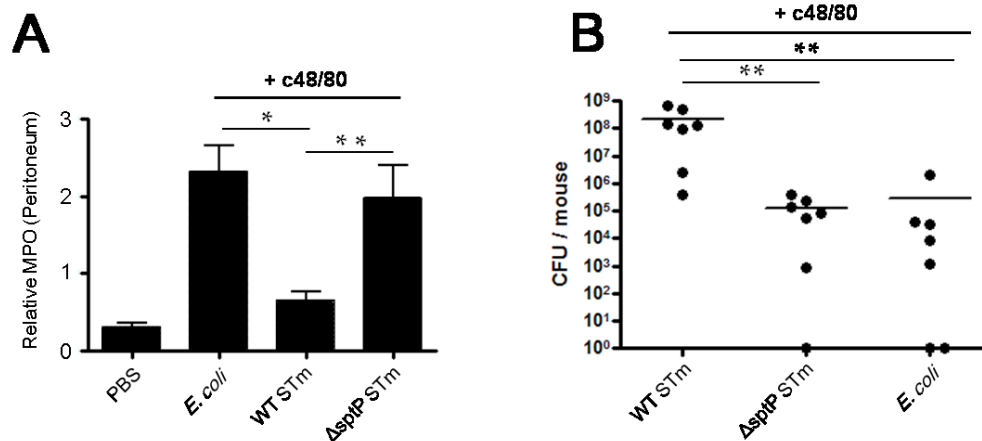




**Figure 27.  $\Delta$ sptP *S. Typhimurium* infected peritoneal MCs evoke a degranulation response to C48/80.**

Mice were I.P. infected with WT or  $\Delta$ sptP *S. Typhimurium*. After 1h, the mice were I.P. infected with C48/80 (20  $\mu$ g/mouse). After 30min, isolated peritoneal MCs were stained with anti-serotonin (red) and anti-pTyr antibodies (blue). Scale bars are 10  $\mu$ m.

As mentioned earlier, MC degranulation leads to neutrophil recruitment and bacterial clearance. Therefore, we investigated whether massive suppression of MCs leads to inhibition of neutrophil recruitment and bacterial clearance. We compared neutrophil responses in the peritoneum of mice following 1 h infection with WT or  $\Delta sptP$  *S. Typhimurium* and 4 h challenge with C48/80. Similar to the results from Figure 26, significantly higher neutrophil responses were observed with  $\Delta sptP$  than with WT *S. Typhimurium* (Figure 28A), and this correlated with greater clearance of  $\Delta sptP$  compared to WT *S. Typhimurium* 6 h after bacterial challenge (Figure 28B). Clearance of  $\Delta sptP$  *S. Typhimurium* was similar to that of *E. coli* that was used as a positive control for neutrophil recruitment and bacterial clearance.

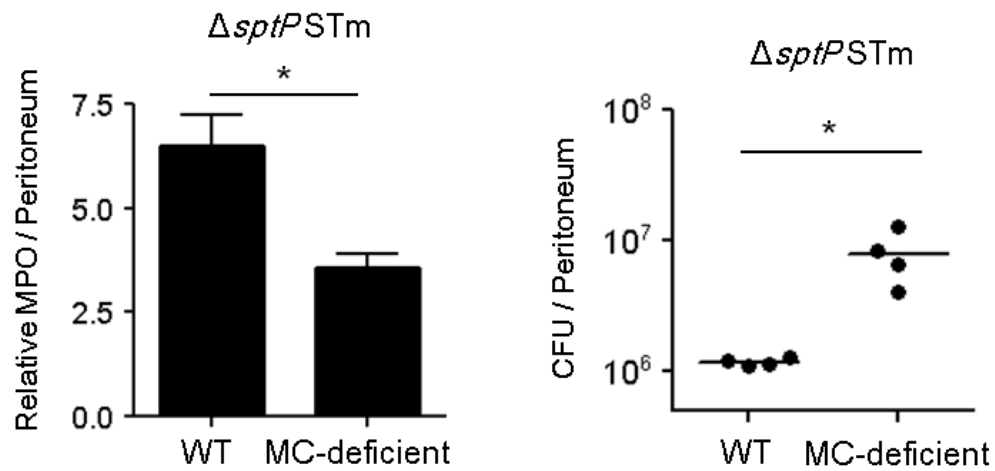


**Figure 28.  $\Delta sptP$  *S. Typhimurium*-infected peritoneal MCs evoke enhanced neutrophil recruitment and bacterial clearance following exposure to C48/80.**

Mice were i.p. infected with WT or  $\Delta sptP$  *S. Typhimurium*. After 1h, the mice were i.p. infected with C48/80 (20  $\mu$ g/mouse). Isolated peritoneal fluid was assayed for measuring MPO after 4h or bacterial counts after 6 h. Mean  $\pm$  SEM, \* $p$ <0.01, \*\* $p$ <0.05.

### ***2.2.18 Enhanced Neutrophil Recruitment and Bacterial Clearance following Infection by $\Delta$ sptP Salmonella***

Given the intrinsic ability of SptP to block MC degranulation, previous studies have not been able to demonstrate a specific role for MCs in combating *S. Typhimurium* infections in WT and MC-deficient mice. Indeed, when we initially compared neutrophil responses and bacterial clearance in WT and MC-deficient mice following intraperitoneal challenge with WT *S. Typhimurium*, we failed to notice any appreciable difference between the two strains of mice (Figure 5A and Figure 6). However, based on our subsequent findings, we sought to demonstrate a potential MC response to *S. Typhimurium* by employing our  $\Delta$ sptP mutant strain. We compared neutrophil responses and bacterial clearance of  $\Delta$ sptP *S. Typhimurium* in WT and MC-deficient mice following peritoneal challenge. Neutrophil responses to  $\Delta$ sptP *S. Typhimurium* were significantly more elevated in WT mice compared to MC-deficient mice (Figure 29, left), and this was accompanied by better clearance of  $\Delta$ sptP *S. Typhimurium* in WT mice 24 h after bacterial challenge (Figure 29, right).

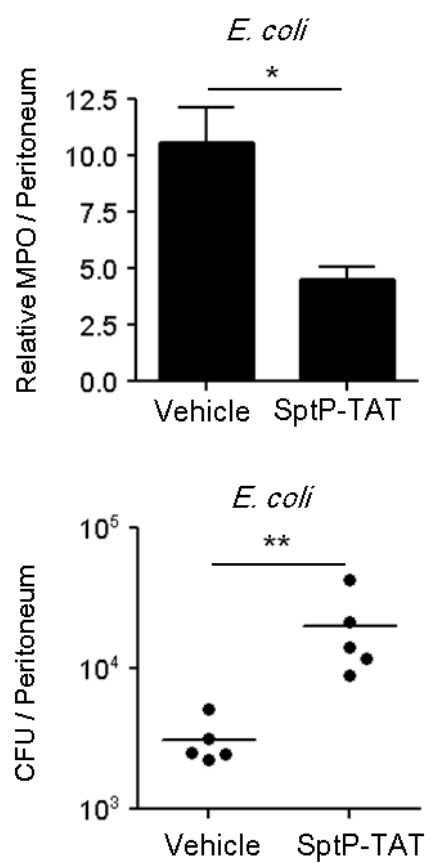
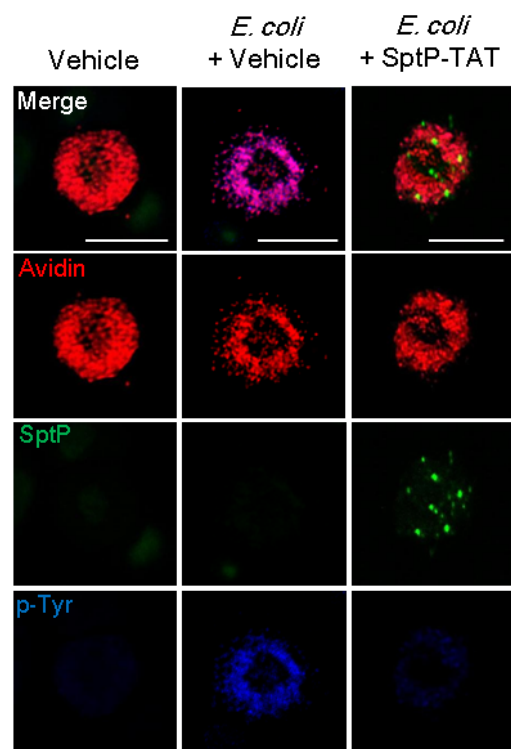
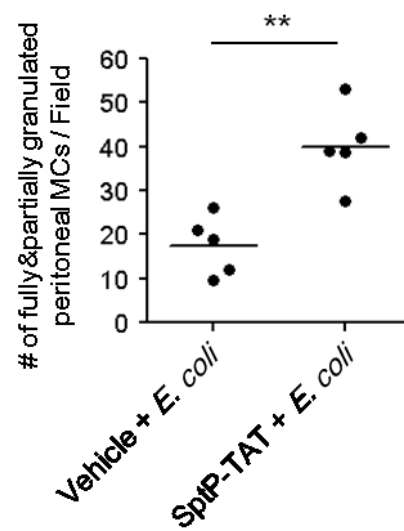


**Figure 29. Enhanced neutrophil recruitment and bacterial clearance following infection by  $\Delta sptP$  *S. Typhimurium*.**

Neutrophil influx (left) and bacterial counts (right) in WT and MC-deficient mice, following i.p. injection of  $5 \times 10^5$  CFU  $\Delta sptP$  *S. Typhimurium*. Mean  $\pm$  SEM, \* $p < 0.05$ .

### ***2.2.19 S. Typhimurium utilizes SptP to inhibit MC-initiated neutrophil influx and bacterial clearance***

To demonstrate the potency of the MC suppressing activity of SptP-TAT, we co-administered recombinant SptP-TAT i.p. with *E. coli* J96, which normally activates MCs, and examined neutrophil recruitment and bacterial clearance. Compared to *E. coli* J96 alone, *E. coli* J96 co-instilled with SptP-TAT evoked lower neutrophil responses and bacterial clearance from the peritoneum (Figure 30A). To verify that this weak neutrophil response was linked to MCs, we examined isolated peritoneal MCs and found that those from mice challenged with *E. coli* J96 with SptP-TAT were non-degranulated (Figure 30B, right column) in contrast to mice challenged with *E. coli* J96 alone (Figure 30B, middle column, quantitated in Figure 30C). Furthermore, these non-degranulated MCs exhibited a limited degree of tyrosine phosphorylation and appeared to have accrued detectable amounts of SptP-TAT in their granule compartments (Figure 30B, right column).

**A****B****C**

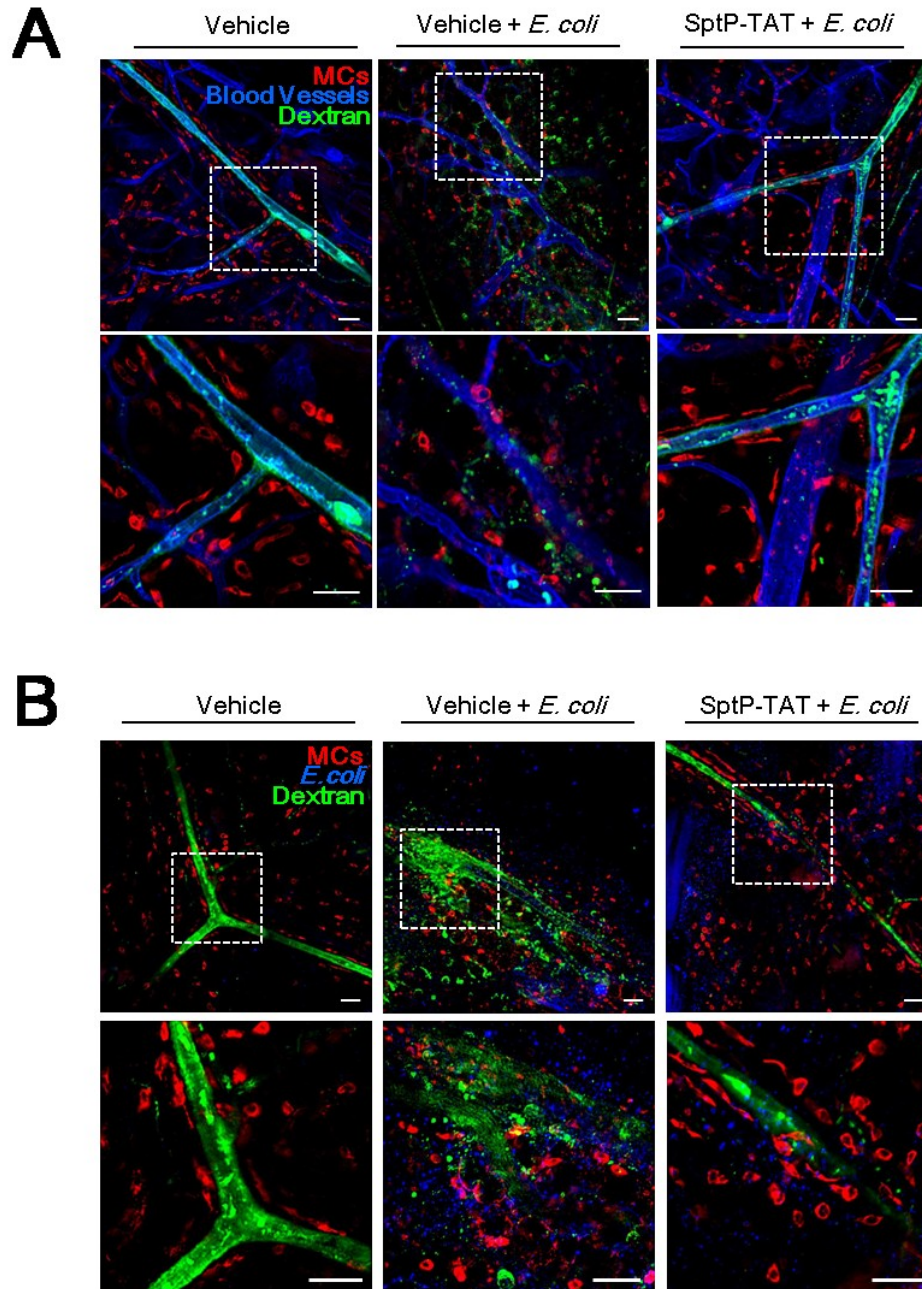
**Figure 30. Administration of SptP-TAT at sites of *E. coli* infection inhibits MC-mediated neutrophil recruitment resulting in impaired bacterial clearance.**

(A) Neutrophil influx (top) and bacterial counts (bottom) in WT mice were measured, after SptP-TAT (100 µg/mouse) or vehicle followed 30 min later with  $1 \times 10^7$  CFU *E. coli*. MPO assays were performed 5 h post-infection and CFUs 24 h post-infection. (B) Morphologies of MCs in the peritoneal cavities of control and SptP-TAT treated mice 3 h following *E. coli* infection. MC granules: avidin (red), SptP-TAT: anti-His<sub>6</sub> (green) and sites of tyrosine phosphorylation: anti-p-Tyr antibodies (blue). Scale bars: 20 µm. (C) Granulated MC numbers in peritoneal lavages of (B) were quantified by counting the number of partially and fully granulated MCs/field (n=5; 5 random chosen fields). Fully degranulated MCs could not be detected.

### ***2.2.20 Inhibition of MC-Mediated Vascular Leakage by SptP-TAT.***

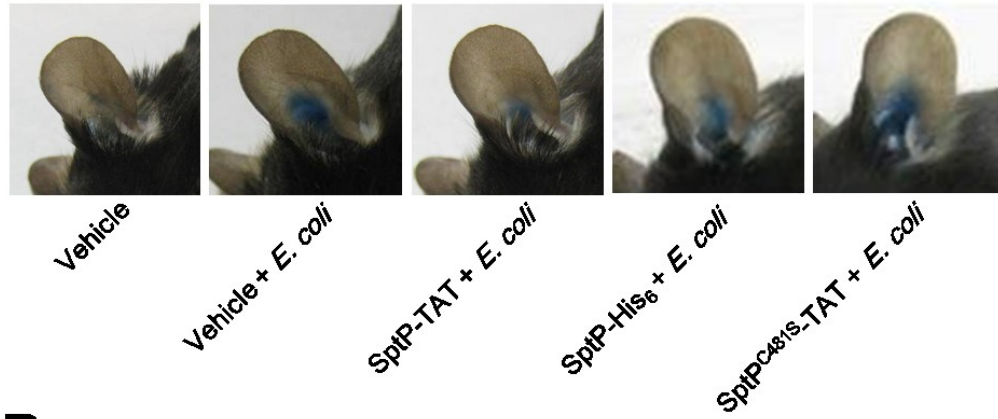
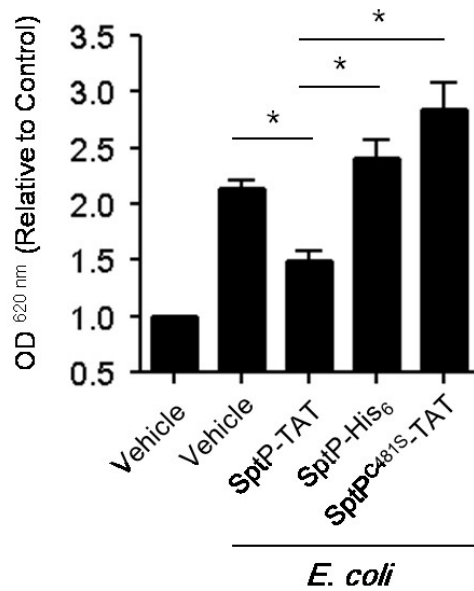
MC-mediated neutrophil recruitment is also associated with vascular leakage of serum components, including complement and antibodies, that can markedly impact pathogen persistence in tissue sites [103,104]. We sought to examine the impact of SptP on MC-mediated vascular leakage by assessing the tissue penetrance of intravenously administered FITC-dextran. Figure 31 shows the whole mount staining of mouse ears that were intradermally injected with vehicle or SptP-TAT followed by *E. coli* J96 infection. Based on the large amount of FITC-dextran in the perivascular space (Figure 31A and B, middle), *E. coli* J96-injected tissue exhibited a high degree of vascular leakage. A high degree of MC degranulation was also observed in this tissue. In contrast, no vascular leakage and MC degranulation was observed in tissue injected with *E. coli* and SptP-TAT (Figure 31A and B, right). A significant reduction in vascular leakage induced by SptP-TAT following *E. coli* J96 infection of mouse ears was quantitatively measured by examining extravasated Evans Blue dye (Figure 32). Additional whole mount staining revealed that SptP-TAT inhibited degranulation following exposure to *E. coli* J96 (Figure 31B) and that this inhibitor could be detected in the cytosol of MCs in infected tissue (Figure 33). Cumulatively, these findings demonstrate that SptP is a potent suppressor of MC-mediated activity, and in the absence of SptP, MCs would likely play an antibacterial role during infection with *S. Typhimurium*.





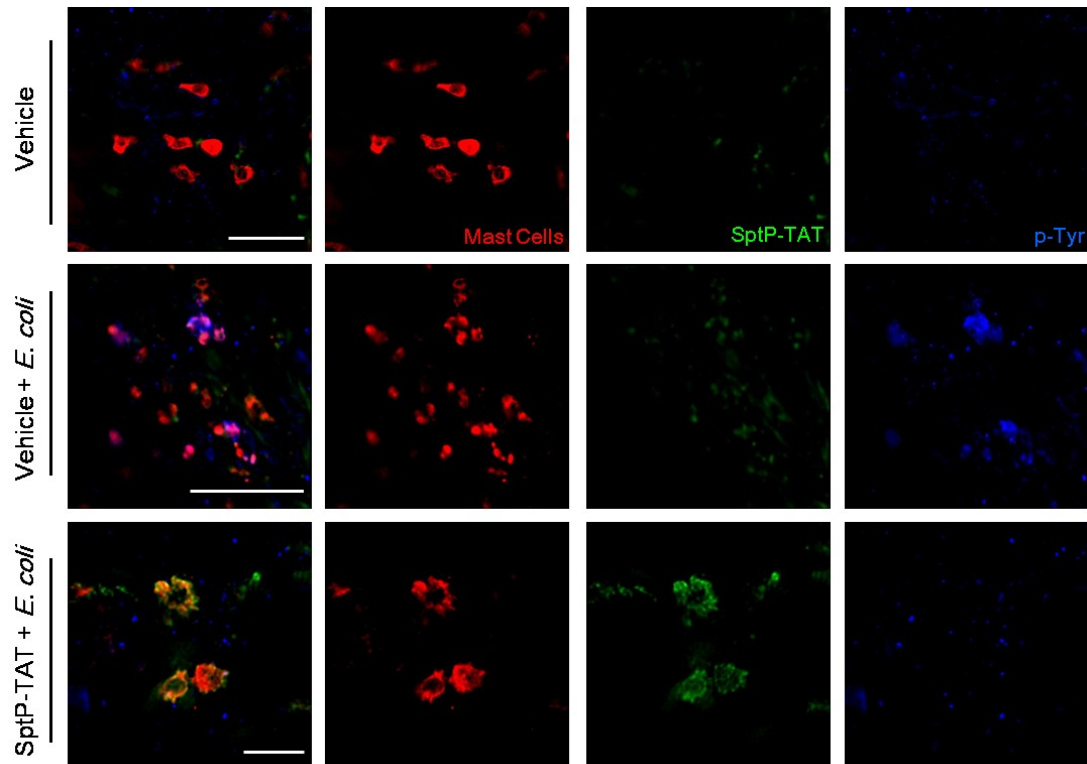
**Figure 31. Vascular leakage resulting from MC degranulation following *E. coli* infection is suppressed by the administration of SptP-TAT.**

(A and B) Mouse ears were injected intradermally with vehicle or SptP-TAT (20  $\mu$ g) and injected 1 h later with  $1 \times 10^6$  CFU *E. coli* J96 at the same site and FITC-dextran (green) i.v. At 1 h post-infection, the ears were dissected, (A) stained with avidin (red) and anti-CD31 (blue), (B) stained with avidin (red) and anti-*E. coli* (blue), and whole-mounted for immunofluorescence microscopy. Scale bars: 50  $\mu$ m.

**A****B**

**Figure 32. SptP-TAT-suppressed MC degranulation and accompanying vascular leakage during *E. coli* infection**

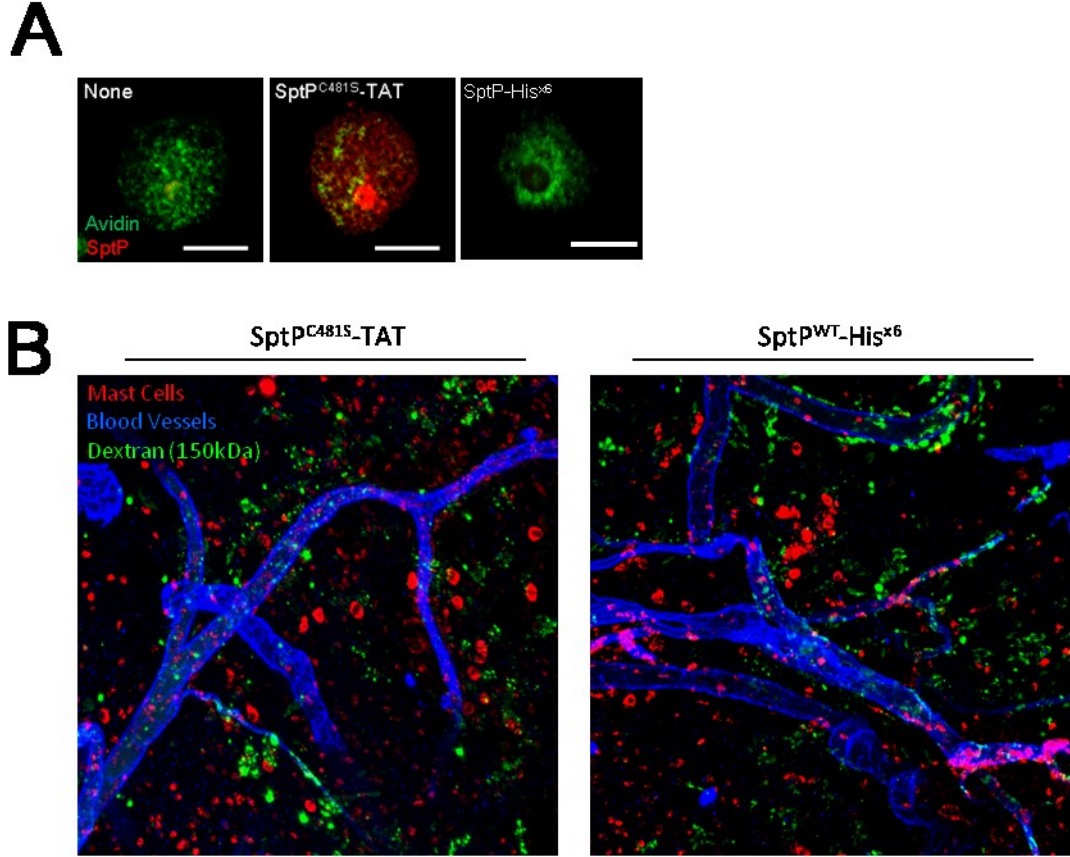
(A) Vehicle or SptP-TAT or SptP-His<sub>6</sub> or SptPC<sup>481S</sup>-TAT was intradermally injected into mouse ears. At 1 h post-infection,  $1 \times 10^6$  CFU *E. coli* were injected at the same site, and Evans Blue dye (0.5%) was simultaneously injected intravenously. At 1 h post-infection, (A) the extravasation of Evans Blue was visualized in infected ears. (B) Vascular leakage was quantified by measuring extracted dye from dissected ears at 620 nm absorbance. Y axis values are relative to negative control. Mean  $\pm$  SD. \* $p < 0.01$



**Figure 33. SptP-TAT inhibited tyrosine phosphorylation signaling and degranulation in MCs.**

The same procedure described in Figure 31 was performed. Avidin (red), an anti-His antibody (green), and anti-pTyr antibody (blue) were used to stain whole ear tissue. The tissue was visualized by confocal microscopy. Scale bar: 50  $\mu$ m.

To exclude the possibility of off-target effect of SptP<sup>WT</sup>-TAT on MCs, we verified whether SptP<sup>C481S</sup>-TAT can penetrate MCs and suppress MC degranulation. The results showed that SptP<sup>C481S</sup>-TAT could penetrate MCs (Figure 34A, middle panel) but failed to suppress MCs and led to significant vascular leakage at the local tissue site (Figure 34B, left panel). Additional control experiments with SptP<sup>WT</sup>-His<sup>x6</sup> showed that penetration of SptP by itself rarely occurred (Figure 34A, right panel) and failed to suppress MC mediated vascular leakage (Figure 34B, right panel).



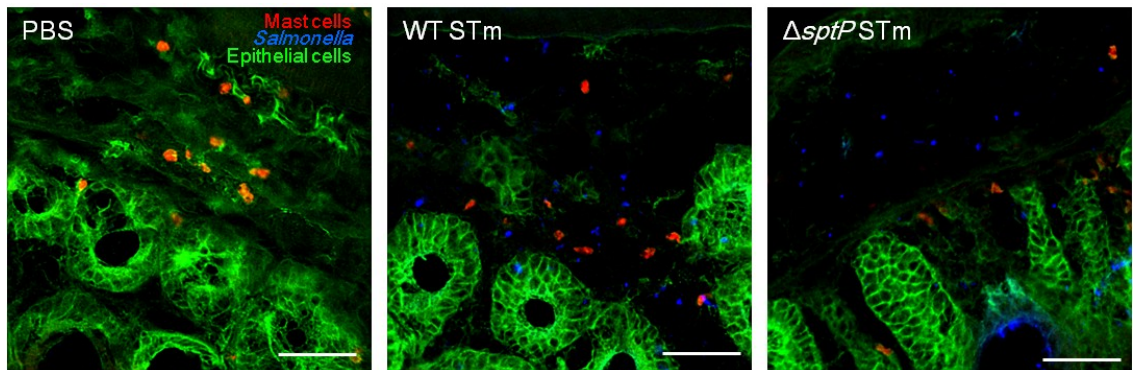
**Figure 34. SptP<sup>C481S</sup>-TAT and SptP-His<sup>x6</sup> failed to suppress MCs *in vivo*.**

(A) BMMCs exposed to SptP<sup>C481S</sup>-TAT or SptP-His<sup>x6</sup> for 4 h. Confocal microscopy of MCs revealed granules probed with avidin (green) and intracellular SptP<sup>C481S</sup>-TAT probed with anti-His<sup>x6</sup> (red). (B) Mouse ears were injected intradermally with SptP<sup>C481S</sup>-TAT (20 µg) or SptP-His<sup>x6</sup> (20 µg) and 1 h later,  $1 \times 10^6$  CFU *E. coli* were injected into the same area of each ear. At the same time a marker of vascular contents, FITC-dextran (green), was intravenously administered. At 1 h post-infection, the ears were dissected and stained to visualize MC granules: avidin (red) and blood vessels: anti-CD31 (blue) antibody. Tissues were whole mounted for confocal microscopy.



### **2.2.21 MC-*S. Typhimurium* Interactions Likely Occur Immediately After the Gut Epithelium is Breached.**

During natural infection, *S. Typhimurium* that successfully breach the gut epithelial barrier typically target the mesenteric lymph nodes, where they proliferate with limited interference from the host immune system [84]. However, before *S. Typhimurium* can reach the draining lymph nodes, the bacteria must circumvent an array of MCs present in the lamina propria of the gut epithelium. Shown in Figure 35 are sections of the cecal epithelium of mice challenged with PBS, WT *S. Typhimurium* or  $\Delta sptP$  *S. Typhimurium*. Numerous fully granulated MCs were seen proximal to the epithelium in PBS or WT *S. Typhimurium*-challenged mice but not in mice challenged with  $\Delta sptP$  *S. Typhimurium* which are incapable of suppressing MCs. Indeed, the apparent absence of fully granulated MCs in the cecum of  $\Delta sptP$  *S. Typhimurium* challenged mice was attributable to extensive MCs degranulation induced by the many mutant *S.*



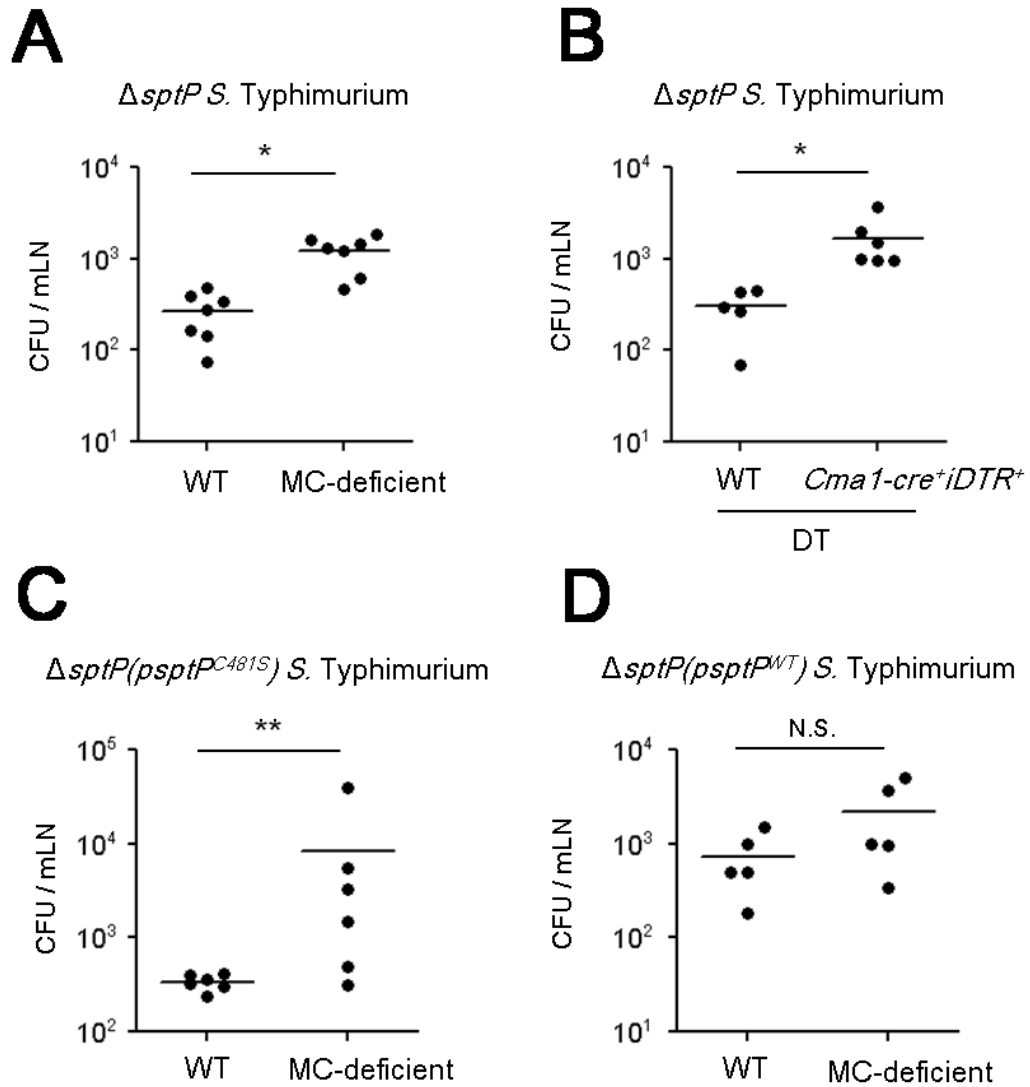
**Figure 35. MC degranulation in the cecum of mice infected with  $\Delta sptP$  but not in WT *Salmonella* infected mice.**

Wild-type mice were pre-treated with streptomycin (20 mg/mouse) for 24 h, followed by oral infection with  $1 \times 10^8$  CFU of WT or  $\Delta sptP$  *S. Typhimurium*. After 12 h, the ceca were dissected, cryo-sectioned and immunostained for MCs: avidin (red), *S. Typhimurium*:  $\alpha$ -*Salmonella* (blue), and epithelial cells:  $\alpha$ -E-cadherin antibodies (green). Shown in the first panel is the cecum of PBS treated mice (control). Shown in the second and third panels are ceca of mice infected with WT or  $\Delta sptP$  *S. Typhimurium*, respectively. Scale bar: 50  $\mu$ m.

Typhimurium found at this site (Figure 35).

We investigated whether these MCs in the lamina propria played a role in controlling bacterial numbers, thus suggesting a plausible role for *S. Typhimurium*-mediated MC suppression during natural infection. To address this question, we assessed the number of bacteria that reached the mesenteric lymph nodes in WT and MC-deficient mice shortly after oral challenge with  $\Delta sptP$  *S. Typhimurium*, which does not suppress MCs. If gut-resident MCs contribute to bacterial clearance, we would expect fewer bacteria to reach the mesenteric nodes in MC-sufficient mice compared to their MC-deficient counterparts. We detected markedly greater numbers of bacteria in the immediate draining lymph nodes of MC-deficient mice 24 h after oral infection with  $\Delta sptP$  *S. Typhimurium* compared to WT mice (Figure 36A). Because some recent studies have questioned the use of *Kit*-dependent MC-deficient mice [25,26,85], we have also included here a recently described *Kit*-independent inducible model of MC deficiency involving *Cma1-cre<sup>+</sup>iDTR<sup>+</sup>* mice [25]. Using this model, we obtained very similar findings regarding the contribution of MCs in the clearance of  $\Delta sptP$  *S. Typhimurium* (Figure 36B). Additionally, we confirmed the contribution of the phosphatase domain of SptP by challenging WT and MC-deficient mice with  $\Delta sptP(psptP^{C481S})$  *S. Typhimurium* (Figure 36C). To confirm that the SptP deletion was responsible for MC-dependent bacterial clearance, we also infected WT and MC-deficient mice with  $\Delta sptP(psptP^{WT})$  *S. Typhimurium* (Figure 36D). Predictably, we observed that the bacterial burden in MC-sufficient mice was now comparable to that of MC-deficient mice. This result is also consistent with the data presented in Figure 5A demonstrating that the WT *S. Typhimurium* burden was comparable in MC-sufficient and MC-deficient mice. Thus, MCs have the potential to regulate the colonization of the mesenteric

lymph nodes, which are among the first sites targeted by pathogens that successfully breach the gut barrier.



**Figure 36. Intestinal MCs reduce the bacterial burden in the mesenteric lymph nodes following oral infection with  $\Delta sptP$  *S. Typhimurium*.**

CFU of mesenteric lymph nodes in streptomycin-treated (A, C, D) WT or MC-deficient mice, (B)  $Cma1-cre^+ iDTR^+$  mice or WT controls after DT injections, following oral challenge with  $5 \times 10^7$  CFU (A, B)  $\Delta sptP$  *S. Typhimurium*, (C)  $\Delta sptP(psptP^{C481S})$  and (D)  $\Delta sptP(psptP^{WT})$  24 h post-infection. \* $p < 0.01$ , \*\* $p < 0.05$ , N.S., not significant.



## 2.3 Discussion

In this study, we have revealed a highly effective mechanism involving the inactivation of MCs employed by *S. Typhimurium* to elude host innate immunity, which are immune surveillance cells strategically located at host-environment interfaces. We demonstrated that unlike *E. coli* J96, which provoked rapid and extensive MC degranulation, *S. Typhimurium* not only failed to elicit a MC degranulation response but also prevented MC responses to a wide array of potent secretagogues such as IgE+antigen, a calcium ionophore and complement revealing a broad and powerful inhibitory activity (Figure 10, 15 and 27). This capacity to block MC responses to other signals is relevant during infection because even though *S. Typhimurium* do not activate MCs *per se*, various endogenous danger signals or alarmins are released at infection sites and can potentially activate MCs [105]. *In vivo* studies in mice revealed that this innate capacity of *S. Typhimurium* to block MC degranulation markedly reduced neutrophil responses and limited bacterial clearance at the infection site. This is in sharp contrast to *E. coli*, which elicited extensive MC degranulation, a strong neutrophil response, and marked bacterial clearance from the site of infection. This capacity to elude early innate immune responses by inactivating peripheral MCs may explain, at least in part, the well-recognized ability of *S. Typhimurium* to avoid clearance by the innate immune system and to disseminate rapidly from the site of infection to distal sites such as the draining lymph nodes and liver [84]. Our observation that *S. Typhimurium* inhibited early neutrophil recruitment is not inconsistent with earlier reports of strong neutrophil responses following *S. Typhimurium* infection [106,107]. In this study, we focused on a narrow window of time during the early stages of the infection process but observed the development of a vigorous MC-independent neutrophil response at sites of infection with WT *S. Typhimurium* after the initial lag period. Our findings imply that the

initial immune response to a pathogen is an important determinant of infection outcome. A delayed neutrophil response at the site of *S. Typhimurium* infection, regardless of its intensity, may have limited protective value because many of the bacteria are either intracellular or have become systemic at that point.

The ability of *S. Typhimurium* to block MC activity could, at least in part, explain why others have not been able to demonstrate a protective role for MCs [86]. Much of our conclusions were based on traditional *Kit* mutant MC-deficient mice; however, since this model of MC deficiency has been recently challenged [25,26,85], we sought to confirm our findings by employing *Kit*-independent *Cma1-cre<sup>+</sup>iDTR<sup>+</sup>* mice in which MC deficiency was inducible. Our findings utilizing this inducible model of MC deficiency corroborated our findings with *Kit* mutant mice providing further validation of our conclusions.

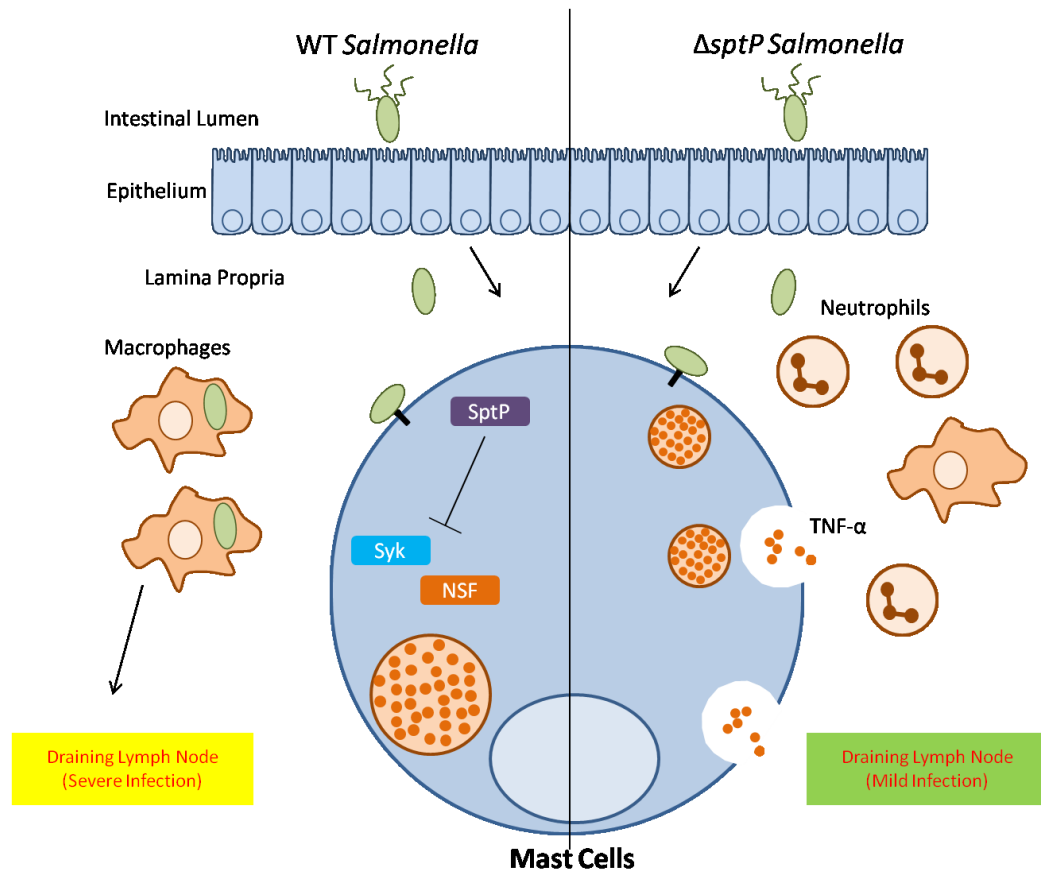
*S. Typhimurium*-mediated suppression of MC degranulation was achieved through the introduction of SptP into the MC cytosol. The suppressive actions of SptP are restricted to the exocytic function of MCs because MCs readily internalize *S. Typhimurium* upon contact. SptP is a SPI1 effector protein with two functional domains. The N-terminal domain functions during pathogen entry into host cells by deactivating Rho GTPases and reversing pathogen-induced cytoskeletal changes following *S. Typhimurium* uptake [91]. The second domain, a C-terminal domain, comprises a protein tyrosine phosphatase reported to function late in the pathogen entry process when *S. Typhimurium* is harbored within late endosomes [108]. In these compartments, it is believed that SptP dephosphorylates a protein that facilitates cellular membrane fusion and protein degradation, enhancing intracellular *S. Typhimurium* replication. The tyrosine phosphatase domain of SptP is also thought to inactivate MAP kinase [109]. Our finding that the tyrosine phosphatase region of SptP mediates MC inactivation represents a

distinct function for this domain, while our work with GTPase-inactive SptP as well as YopH, which lacks a GTPase domain, suggests that GTPase activity is for the most part dispensable for MC suppression. A comparison of the primary structure of SptP with multiple tyrosine phosphatases found in the cytosol of MCs reveals high degree of homology [90], implying multiple targets for this phosphatase. Morphological studies of MCs employing pTyr-specific antibody probes revealed that degranulation was closely associated with phosphorylation events at the cell surface as well as at intracellular sites proximal to cytosolic granules. Phosphorylation of these proteins at both sites did not occur in the presence of SptP, suggesting that SptP acted at multiple sites and on multiple targets. By examining *S. Typhimurium*-infected MCs before or after activation with various secretagogues, we have been able to identify at least two distinct substrates for SptP. Immunoblot analyses of *S. Typhimurium*-infected MCs and control MCs following IgE mediated activation revealed one of the intracellular targets of SptP to be Syk, which is a member of the Syk/Zap-70 family and is a substrate found early in the signaling cascade. Microscopic and immunoprecipitation studies of MCs infected with WT *S. Typhimurium* also revealed that shortly after infection MC granules spontaneously coalesced forming oversized granules that failed to be expelled upon subsequent exposure to potent secretagogues. This phenomenon was not observed with  $\Delta sptP$  *S. Typhimurium*, indicating that it was an SptP-mediated activity. We have linked this intergranular fusion to SptP-mediated dephosphorylation of NSF, a fusion protein whose functions include the prevention of homotypic intergranular fusion [24,93].

Although a number MC-inactivating compounds derived from various microbes have been identified, including the toxic fungal metabolite gliotoxin [110,111], ES-62, a glycoprotein secreted by filarial nematodes [112], FK506 from *Streptomyces tsukubaensis* [113], and

cyclosporine A from *Tolypocladium inflatum* [114], these do not, for the most part, appear to be relevant to the pathogenesis of infection. Here, Our studies reveal that two highly successful host-adapted pathogens, *S. Typhimurium* and *Y. pestis*, share a powerful mechanism to suppress MC degranulation, suggesting that MC inhibition is an important virulence determinant. MCs are among the first immune cells encountered by these pathogens after they traverse the epithelial barrier. They are relatively abundant in dermal tissue, where *Y. pestis* is localized following injection by fleas, and are also common in the gut epithelium below Peyer's patches as well as in the lamina propria, where they can encounter *S. Typhimurium* invading through M cells or gut epithelial cells [115]. Orally-administered *S. Typhimurium* that successfully breach the epithelial barrier traffic to the mesenteric lymph nodes, a process likely to be influenced by MC interactions. Indeed, significantly higher numbers of the  $\Delta sptP$  mutant were present in the mesenteric nodes of MC-deficient mice following oral infection, implicating mucosal MCs as primary responders to *S. Typhimurium* breaching the gut epithelium (Figure 37).

MCs and MC-like cells are found in a wide range of animal species including less evolved species that are devoid of an adaptive immune system. In recent years, it has become clear that MCs play a key role in modulating innate immune responses to various bacteria, parasites, viruses and fungi, suggesting that their *raison d'être* is the modulation of immediate and nonspecific immunity to microbial challenge. In view of this, it is not surprising that certain virulent pathogens would have evolved special mechanisms to inactivate these cells to prevent or delay host immune responses and promote their pathogenesis.



(Ando T. and Kawakami T. (Preview) [116])

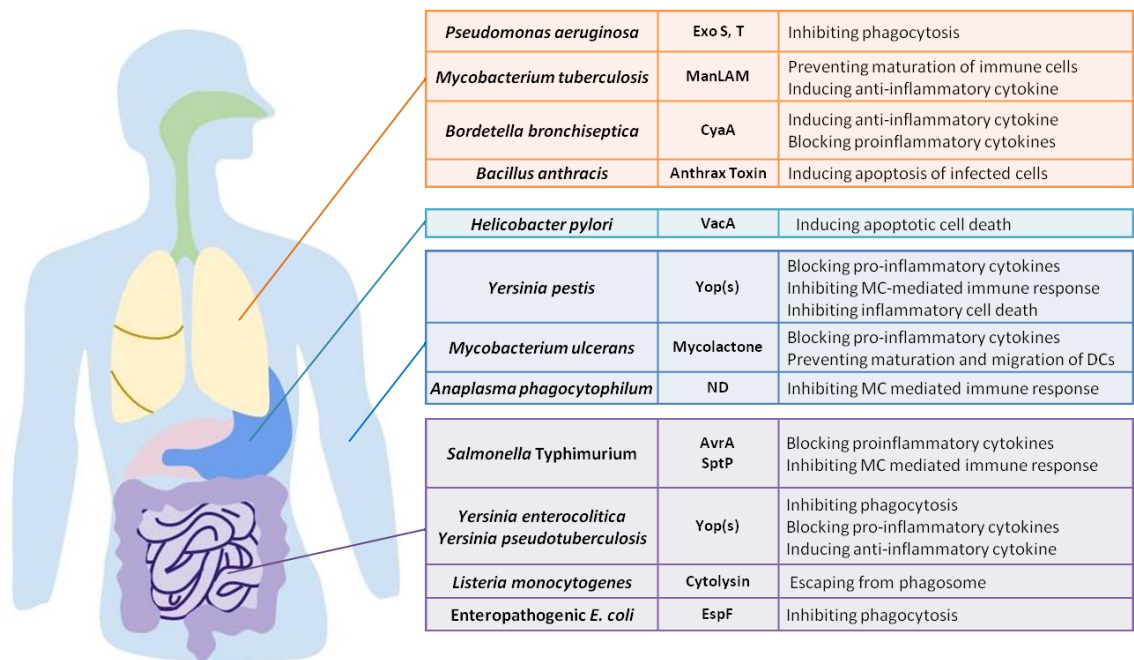
**Figure 37. Model depicting mechanism of MC Suppression by SptP derived from *S. Typhimurium*.**

(Left) WT *S. Typhimurium* secretes SptP into MC to suppress MC activation by dephosphorylating Syk and NSF. Reduced neutrophil recruitment leads to severe infection. (Right)  $\Delta sptP$  *S. Typhimurium*, which lack SptP, fails to suppress MC degranulation and leads to the early recruitment of neutrophils to clear bacteria at an early stage of infection [116].

### 3. Perspective

In this thesis I have described how pathogenic *Salmonella* have evolved powerful mechanisms to inactivate MCs which are key immune surveillance cells located in the gut as well as other mucosal sites. I have demonstrated that *Salmonella* achieve this feat by secreting a tyrosine phosphatase, SptP, directly into the cytosol of MCs. The translocation of SptP into MCs occurs via the T3SS which injects various *Salmonella* effector proteins via a syringe-like structure that appears to traverse the plasma membranes of MCs and other target cells. Once introduced into the MC cytosol, SptP was found to target multiple substrates involved in MC degranulation. One of the substrates was Syk a key conduit of early signaling from receptors located in the MC membrane and the other was NSF a critical mediator of the final steps of MC degranulation. Interestingly, SptP was found to dephosphorylate NSF resulting in premature intergranular fusion in MCs while it prevented phosphorylation of Syk following MC exposure to *Salmonella*. Both of these activities completely blocked the MC degranulation response to *Salmonella* but remarkably had no effect on the endocytic activities of the cell. Consequently infecting *Salmonella* were able to readily infect MCs and seek intracellular refuge in the same immune cells whose function it is to warn the host of the infection. Microscopic examination of the cecum of mice following oral challenge with *Salmonella* revealed that the bacteria encountered MCs as soon as they had circumvented intestinal epithelium in cecum (Figure 35). The importance of SptP in modulating the outcome of the *Salmonella*-MC encounter could be gauged by the finding that the translocation of sptP mutant *Salmonella* from the gut into the draining mesenteric lymph nodes of MC deficient mice was markedly higher than that of WT mice (Figure 36A).

While the focus of this thesis has been on MC suppression by *Salmonella* there are other pathogenic bacteria capable of targeting immune surveillance cells such as dendritic cells (DCs) and macrophages (Mφs) for suppression of functional activities. Not surprisingly, the strategies involved by these pathogenic bacteria appear, for the most part, distinct from what I have described here. Shown in Figure 38 are some of diverse strategies and bacterial components employed by various pathogens to suppress immune surveillance cells [117]. In the following section, I will describe briefly how two other well known host adapted pathogens, the Gram negative pathogen *Yersinia pestis* (and related species) and the Gram positive *Mycobacterium tuberculosis* suppress DCs and macrophages.



**Figure 38. Pathogenic infection in human body.**

### 3.1 Immune suppressive abilities of *Yersinia* and *Mycobacteria* species.

*Yersinia pestis* is a Gram-negative and facultative bacteria which is the causative agent of plague [118]. The mortality associated with this pathogen can reach 20-50 % even with antimicrobial treatment [119]. The virulence of this pathogen is associated with its multifaceted approach to inactivating key immune surveillance cells such as MCs, DCs and macrophages. As already indicated, *Y. pestis* suppresses MCs by directly injecting YopH into the cytosol of these immune cells. However, *Yersinia spp.* also target Mφs and DCs for inactivation. This is achieved by the translocation of various effector proteins into the cytosol of the target cells via *Yersinia*'s T3SS. These effector proteins inactivate substrates in various signaling pathways involved in the secretion of key proinflammatory cytokines and chemokines. Through the secretion of YopJ, an acetyltransferase, into the cytosol of Mφs, *Yersinia spp.* are able to block the phosphorylation ability of MAPKK (Mitogen-activated protein kinase kinase). Thus leads to the blockage of the NF-κB signaling pathway, which in turn is critical for the secretion of many key proinflammatory cytokines such as TNF-α, IL-12, 15, and 18 [120,121]. While certain effectors of *Yersinia* such as YopJ block the production by macrophages of proinflammatory mediators, other bacterial factors appear to trigger secretion of the immunosuppressive cytokine, IL-10. *Y.*

*pseudotuberculosis* and *Y. enterocolitica* can induce IL-10 production from macrophages through the release of virulence (V)-antigen (LcrV). When LcrV binds to toll-like receptor 2 (TLR2) on Mφs, IL-10 is secreted and functions as inducing distinct tolerance [122,123]. IL-10 has a potent effect in suppressing the production of various proinflammatory cytokines from surrounding cells which is beneficial for the spread of *Yersinia* and also in preventing the development of the



adaptive immune response [122,124]. LcrV also induces IL-10 production in DCs and activates the JNK signaling pathway inducing these infected DCs to differentiate.

In addition to suppressing the production of proinflammatory cytokines from DCs and Mφs, *Yersinia* also appears capable of blocking uptake by the same phagocytic cells. Through simultaneous injection of YopT, YopE, and YopH, *Yersinia* blocks various stages of the phagocytic process at the same time. First, the cysteine protease activity of YopT specifically cleaves Rho GTPases to cause depolymerization of actin filaments and disruption of the actin cytoskeleton [125-128]. Second, YopE activates the GTPase-activating protein (GAP) to down-regulate the Rho family members such as Cdc42, RhoA and Rac1 [129] which also disrupts the cellular cytoskeleton [130]. Third, YopH dephosphorylates the Fyn-binding protein (FYB) to deform the peripheral focal complexes [131] needed for bacterial uptake. Thus, through the secretion of multiple effectors *Yersinia* can negatively regulate the functions of key immune surveillance cells in the microenvironment that it occupies in the host permitting bacterial colonization and spread within the host.

*Mycobacterium tuberculosis* causes tuberculosis, one of the top 10 causes of human morbidity and mortality worldwide. If tuberculosis patients are not treated, fatality rates can reach up to 50 % [132]. Although different *Mycobacterium* spp. causes distinct diseases, they tend to share many virulence mechanisms. For example, *M. tuberculosis*, *M. leprae* and *M. ulcerans* all seem to suppress mediator release from immune surveillance cells which they interact with. They typically also invade and survive intracellularly within these cells. In their intracellular niches these bacteria can avoid the host's immune defenses as well as antibiotic treatment resulting in chronic infections.

*M. tuberculosis* which primarily targets Mφs but also infects DCs suppresses these cells, at least partially with a mannose-containing glycoprotein (ManLAM) that is a mycobacterial cell wall component. ManLAM activates the DC-SIGN signaling pathway which prevents DC maturation [133] resulting in limited DC trafficking to draining lymph nodes and limited adaptive immune responses. Activation of the DC-SIGN pathway results in the activation of the serine/threonine kinase Raf-1. This signaling substrate acetylates NF-κB subunit p65 resulting in enhanced secretion of IL-10 which is strongly anti-inflammatory [134]. *M. tuberculosis* persist in the host by infecting and replicating in phagosomes of Mφs. Infected Mφs form granulomas which serve to encapsulate bacteria within host tissue. Promoting intracellular survival are several secreted virulence proteins such as SapM, a lipid phosphatase, which dephosphorylates phosphatidylinositol 3-phosphate (PI3P) and recruits certain specific proteins into the phagosome in order to avoid acidification and subsequent fusion with lysosomes [135-137]. The phagosome of *M. tuberculosis* has diminished vacuolar H<sup>+</sup>ATPase and low level of lysosomal hydrolase which is favorable for bacterial survival. In DCs, prevention of phagosome maturation severely limits antigen presentation resulting in limited adoptive immune responses in the host [138-140]. Cumulatively, due to their distinct and powerful immunosuppressive actions on DCs and Mφs, mycobacteria are able to successfully establish chronic infections in the host which are very difficult to eradicate.

### **3.2 Coopting MC suppressing properties of bacteria for therapy.**

As indicated earlier in this thesis, MCs can be prominent players in various chronic and harmful inflammatory disorders such as asthma, allergy, psoriasis, arthritis, inflammatory bowel diseases, etc. When more is known regarding how different microbes suppress MCs, it is conceivable that certain microbes or their products can be utilized to ameliorate some of these

chronic inflammatory diseases. For example, it is conceivable that administering cell-permeable forms of SptP into inflammatory sites will impair mediator secretion from MCs and even other immune cells to abrogate inflammation. Its potential usage could be comparable to the administration of Botox, a neurotoxin derived from *Clostridium botulinum* into human facial skin to reduce wrinkle formation [141,142]. The toxin reduces abnormal muscle contraction by blocking release of acetylcholine from neurons, which normally signals muscle contraction. Importantly the effect of Botox can last several months and therefore the frequency of its administration is limited. With the therapeutic success of even a highly toxic agent such as Botox, there is currently great interest in the therapeutic use of other bacterial products which are much less toxic [143].

Some microorganisms or their products are already being used clinically for therapy, unrelated to their MC-suppressing properties, but when their MC suppressing property is also considered, they could have additional clinical uses. For example, the *Lactobacillus* and *Bifidobacterium* species are frequently being applied as probiotics in patients with inflammatory diseases in the gut. In view of the capacity of probiotic bacteria in suppressing IgE-mediated MC responses [144-146], these bacteria or their products may potentially also be used to temper the pathology associated with allergic inflammation. The fungal metabolite Cyclosporin A is routinely used as a potent nonsteroidal immunosuppressive drug in patients following organ transplant [147]. In view of its potent MC suppressing actions [147], this drug could be repurposed for the treatment of various inflammatory disorders where MCs play a key part. The innate capacity of various microbes to suppress MC function may therefore have significant therapeutic promise.

### **3.3 Concluding Remarks**

For many decades the study of bacterial pathogenesis has centered around virulence factors that promote bacterial adherence and colonization of mucosal surfaces as well as factors that promote bacterial entry and survival in various host cells. It is only recently that the immunomodulatory role of bacterial pathogens was recognized. That successful pathogens need to repress or avoid the host's wide array of innate and adaptive immune responses in order to survive is now being recognized as a key virulence trait. Through investigating the various strategies and the underlying molecular basis for bacteria-mediated immune suppression or avoidance, it will be possible to develop appropriate counter measures or vaccines to combat microbial pathogens in the future. These strategies will become even more important with the overuse of antibiotics and the resulting emergence of multi-resistant bacteria in our midst.

## 4. Materials and Methods

### 4.1 Bacterial strains and culture.

*S. Typhimurium* SL1344, SL1344-derived Tn5 transposon insertion mutants in *SPI1*, *SPI2*, and *SPI1&2* were gifts from Dr. Alejandro Aballay, Duke University. SB300, and its *sptP* deletion mutant, SB749, were gifts from Dr. Jorge E. Galán, Yale University.  $\Delta sptP(psptP^{WT})$ ,  $\Delta sptP(psptP^{C481S})$ , and  $\Delta sptP(psptP^{R209A})$  were generated in this study. Other strains used include *E. coli* J96 and CI5, a Gram-positive clinical isolate *Staphylococcus aureus* strain 54 (Duke University Medical Center), and a WT laboratory strain of conditionally virulent and naturally occurring *Yersinia pestis* KIM5. Bacteria were grown for 15 h, non-shaking, in Luria-Bertani (LB) (Gibco) broth at 37°C. 25 µg/ml kanamycin was added for transposon mutants, 100 µg/ml streptomycin for SB300 and SB749, and 100 µg/ml carbenicillin for complemented strains.

### 4.2 Mice.

6- to 8-week-old C57BL/6 mice were purchased from the National Cancer Institute. MC-deficient Wsh mice ( $Kit^{W-sh}/Kit^{W-sh}$ ) and their congenic littermate controls were purchased from Jackson Laboratories. To induce MC depletion, eight-week-old  $Cma1-cre^+iDTR^+$  (a gift from Dr. Axel Roers, University of Technology, Dresden) and wild-type littermates received five i.v. injections of 100 ng DT per mouse in a week. Mice were housed in the Duke University Vivarium and provided food and water *ad libitum*. All experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

### 4.3 Animal infections and CFU counts.

Mice were infected intraperitoneally with the indicated CFU of various strains of *S. Typhimurium*, or *E. coli* J96 in 100 µl of sterile PBS. At the indicated times, mice were euthanized,

5 ml of sterile, ice-cold PBS was injected into the peritoneal cavity, and the abdomen was gently massaged. The collected peritoneal fluid was analyzed for MC degranulation, neutrophil influx, or bacterial counts. The detailed procedure of oral infection to streptomycin-pretreated mice was described [148]. Briefly, mice were pretreated with 20 mg streptomycin prior to infection with  $5 \times 10^7$  CFU of  $\Delta sptP$  or its complemented mutant *S. Typhimurium*. At various times, mice were euthanized and mesenteric lymph nodes homogenized in 0.1% Triton X-100 per PBS. Lysates were plated on MacConkey agar with or without 50  $\mu$ g/ml streptomycin and incubated overnight at 37°C for CFU.

#### **4.4 Cell culture.**

RBL-2H3 cells were grown in MEM (Gibco) supplemented with 15% FBS (GEMINI Bio-Products). BMMCs were prepared by flushing femurs from C57BL/6 mice with cRPMI (10% FBS, 100 U/ml Pen Strep, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids (all from Gibco)). LAD2 cells were grown in StemPro-34 (Gibco) (2.6% StemPro-34 nutrient supplement, 2 mM L-glutamine, 100 U/ml Pen Strep, and 100 ng/ml SCF (R&D systems)). Flushed bone marrow were cultured for 12 weeks in cRPMI containing 5 ng/ml IL-3 and 5 ng/ml SCF (R&D systems). All cells were culture at 37°C in a humidified water-jacketed incubator under 5% CO<sub>2</sub> / 95% air atmosphere.

#### **4.5 $\beta$ -hexosaminidase assay.**

RBLs or BMMCs or LADs were plated in 96-well plates on the day before the experiment. For experiments with IgE-stimulation, cells were sensitized overnight with 1  $\mu$ g/ml IgE (BD Biosciences). RBLs were exposed to bacteria as indicated in Tyrode's buffer for 30 min. In secondary stimulus experiments, cells were stimulated either with ionomycin (1  $\mu$ g/ml) or C48/80 (10  $\mu$ g/ml) or C5a (0.5 ng/ml) or TNP-OVA (10 ng/ml) (all from Sigma) or anti-IgE

antibody in Tyrode's buffer at 37°C. The detailed procedure of  $\beta$ -hexosaminidase assay was described previously [51].

#### **4.6 Microscopy.**

Peritoneal lavage fluids were cytopun by using Cytospin3 (Shandon) on Superfrost Plus glass slides (VWR). Mesentery was carefully dissected and dried on slides. To stain MCs, freshly prepared Carnoy's fixative (ethanol, chloroform, and glacial acetic acid) and 0.5% Toluidine blue were used. Fully degranulated MCs could not be detected, because there were no granules left in MCs to be detected. Peritoneal lavage cells or BMMCs were cytopun and fixed in 4% PFA. 0.1% saponin (Sigma) in 1% BSA in PBS was used to permeabilize cells and dilute antibodies. For whole mount staining of ear tissue, dissected ears were fixed in 4% PFA (2 h) and permeabilized with 0.3% Triton X-100 in 1% BSA in PBS containing 5% goat serum. Cells or tissues were incubated with primary antibodies: antibody to His<sub>6</sub> (Roche), *E. coli*, p-Tyr, CD31 (BD Biosciences), and serotonin (DakoCytomation). The secondary antibodies with FITC or Cy3 or A647-conjugated anti-mouse, A647-conjugated anti-rabbit, DyLight 649-conjugated anti-rat, Cy3-conjugated anti-mouse antibodies (Jackson Immuno Research) and TRITC or FITC conjugated avidin (Sigma) was incubated. Nikon ECLIPSE TE200 microscope (Nikon) was used to observe confocal images. To prevent spectral overlapping between individual fluorescent emission signals, a channel-series approach of EZ-C1 Nikon software was utilized.

#### **4.7 Myeloperoxidase activity assay.**

Cells derived from peritoneal lavage were resuspended in hexadecyltrimethyl ammonium bromide (Sigma) in phosphate buffer and lysed by multiple freeze-thaw cycles. Lysed cells were centrifuged and supernatant was mixed with o-dianisidine and hydrogen

peroxide in phosphate buffer. Activity was measured during 1 min interval three times at 450 nm.

#### **4.8 Construction of SptP plasmids and transfected cell lines.**

Full-length SptP was amplified from SL1344 and cloned into pWSK29, pLEGFP-C1 (Clontech), and pET 28b TATv1 (a gift from Dr. Steven F. Dowdy, University of California, San Diego). *psptP<sup>C481S</sup>*, *psptP<sup>R209A</sup>*, EGFP-SptP<sup>C481</sup>, and SptP<sup>C481S</sup>-TAT plasmids were constructed by site-directed mutagenesis using PCR. Sequences of PCR primers were described in Table 3. For complementation,  $\Delta$ *sptP* *S. Typhimurium* was transformed by electroporation with 100 µg/ml carbenicillin for selection. For stable expression of EGFP-SptP<sup>WT</sup> or EGFP-SptP<sup>C418S</sup>, viral particles were produced from the AmphoPack-293 cell line (BD Biosciences) and infected into RBLs with geneticin selection (Invitrogen).



**Table 3. PCR Primer Sequence**

Plasmids	Primers	
pWSK29	<i>psptP</i>	5'-CGG GAT CCC ATG CTA AAG TAT GAG GAG AG-3'
		5'-CCC AAG CTT TCA GCT TGC CGT CGT CAT AA-3'
	<i>psptP<sup>C481S</sup></i>	5'-ATA AGC ATT TAC CGA TGA TTC ATT CCC TGG GC-3'
		5'-CCG GTT CTT CCC ACT CCG CCC AGG GAA TGA AT-3'
	<i>psptP<sup>PR209A</sup></i>	5'-GCT TCA GGT AAC GGC CCG CTG GCC TCA TT-3'
		5'-TCT GTA AAT TCG TCA TTA ATG AGG CCA GC-3'
pLEGFP-C1	<i>psptP</i>	5'-CCA AGC TTC GAT GCT AAA GTA TGA GGA GAG AA-3'
		5'-CGC GGA TCC GCG TCA GCT TGC CGT CGT CAT AAG C-3'
pET 28b TATv1	<i>psptP</i>	5'-CGC GGA TCC GAT GCT AAA GTA TGA GGA GAG AAA AT-3'
		5'-CCC AAG CTT TCA GCT TGC CGT CGT CAT AAG CAA C-3'

## 4.9 SptP-TAT purification and Column Chromatography

SptP-TAT and SptP<sup>C481S</sup>-TAT were expressed in BL21-Gold (DE3) competent cells (Agilent Technologies) grown in LB media, purified by His GraviTrap TALON (GE Healthcare life Sciences) chromatography and dialyzed in PBS with 0.1% 2-mercaptoethanol. After concentration, protein samples were stored in 10% glycerol at -80°C. Vehicle was PBS with 0.1% 2-Mercaptoethanol and 10% glycerol. To separate proteins from *Salmonella* culture media, gel-filtration column was utilized. *Salmonella* cultured media was collected and concentrated by molecular cut-off column

(10kDa). The concentrated culture supernatant was loaded onto the gel-filtration column which was packed with sepharyl S-200 resin. Eluted fractions were collected and analyzed for assays.

#### **4.10 Statistical analysis.**

Results were analyzed with one-way ANOVA and Tukey's post-test or two-way ANOVA or the Mann-Whitney t-test as appropriate with Prism (GraphPad) software. All *in vitro* experiments were repeated with three independent conditions. Differences between groups were considered significant at  $p < 0.05$ . All error bars represent SEM.

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## Biography

Hae Woong Choi was born on September 30th, 1978 in Pusan, South Korea. He received a Bachelor degree in Life Science and a Master degree in Biochemistry from Korea University in Seoul in February 2005 and in February 2007, respectively. In the fall of 2008, he began his Ph.D. study in the program of Cellular and Molecular Biology at Duke University in Durham, North Carolina in USA. At the second year of his doctoral study, he joined the Department of Pathology and began his dissertation research supervised by Dr. Soman N. Abraham in the Department of Pathology, Immunology and Molecular Genetics and Microbiology. His publications are as follows:

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